MATERIAL DESIGN OF CELL SCAFFOLDS AND BIO-SIGNALING MOLECULES RELEASE FOR TISSUE REGENERATION

YU KIMURA

2009

Dedicated to

my parents

Yoshitaka Kimura

Shigeyo Kimura

CONTENTS

Chapter 2

Effect of material properties on regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor combined with human preadipocytes

Chapter 3

Effect of chemical composition of scaffold materials on proliferation and adipogenic differentiation of human preadipocytes

PART II

REGENERATION OF VARIOUS TISSUES BY CELL SCAFFOLD AND RELEASE MATERIALS OF BIO-SIGNALING MOLECULE WITHOUT CELLS

Chapter 5

In situ regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor

Chapter 6

Regeneration of capillary vessels by gelatin hydrogels containing stromal cell-derived factor (SDF)-1

Chapter 7

Regeneration of bone tissue by gelatin hydrogels containing bone morphogenetic protein (BMP)-2

ABBREVIATIONS

αMEM	Minimum essential medium alpha modified
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
DAB	3,3'-Diaminobendizine
DDS	Drug delivery system
DDW	Double distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FBS	Fetal bovine serum
GA	Glutaraldehyde
GFP	Green fluorescent protein
GPDH	Glycerol-3-phosphate dehydrogenase
GTR	Guided tissue regeneration
HE	Hematoxylin and eosin
IEP	Isoelectric point
PBS	Phosphate-buffered saline
PEG	Poly(ethylene glycol)
PlGF	Placental growth factor
PP	Polypropylene
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SD	Standard deviation
SDF	Stromal cell-derived factor
SSC	Sodium citratre-buffered saline solution
TRITC	5(6)-Tetramethyl-rhodamine isothiocyanate mixed isomer

GENERAL INTRODUCTION

Advanced surgical therapies currently available, such as reconstructive surgery and organ transplantation, have undoubtedly saved and improved many lives of patients, but still have technical and methodological limitations in clinic. For the reconstructive surgery, medical devices cannot completely substitute biological functions even for a single tissue or organ, and consequently cannot prevent the progressive deterioration of injured tissue. For the organ transplantation, the shortage of donor tissues or organs is a serious problem. In addition, the permanent medication of immunosuppressive agents often causes severe side-effects, while virus infection from the donor tissues and organs is not completely ruled out. In these circumstances, a trial of novel therapeutic strategy in which diseases are therapeutically treated based on the natural healing potential of body itself to regenerate autologous tissues, has been explored. The strategy is called tissue regeneration therapy [1]. There are two approaches to achieve the tissue regeneration; cell transplantation and tissue engineering. In the former case, tissue regeneration is realized by the transplantation of cells with the high potentials of proliferation and differentiation. The tissue engineering is a biomedical technology or methodology to create a local environment of cells which assists and enhances their proliferation and differentiation, resulting in cell-based tissue regeneration. The essential concept of tissue engineering was originally indicated by R. Langer and J. Vacanti [2, 3]. They propose in the article that three components are required to induce tissue regeneration: cells or the substrate and nutrient of cells to accelerate their

proliferation and differentiation. At present, the latter two components are being investigated as the cell scaffold and bio-signaling molecules.

Cells used for tissue regeneration are classified to three types of characters [4]: matured cells of terminal differentiation, precursor cells which can differentiate to the matured cells with a specific function, and stem cells which can differentiate to many lineages of matured cells. From the therapeutic viewpoint, it is practically difficult to obtain matured cells with a good quality and sufficient number to transplant by the *in vitro* culture methods. In addition, only by transplanting one type of matured cells, it is biologically impossible to achieve the organization of diverse tissue architectures including vasculature or cell polarity. Based on this, precursor or stem cells which are expected to have higher potentials for tissue organization, have been mainly investigated and applied for tissue regeneration [5-8]. Generally, the cells are proliferated *in vitro*, and then transplanted into the site to be regenerated with or without the cell scaffold to achieve cell-based tissue regeneration. In addition to the cell transplantation, it is promising for cell-based tissue regeneration to make use of cells which are originally present in the body. Recent reports have revealed that hematopoietic progenitor [9-14] and mesenchymal progenitor cells [15-17] are circulated and accumulated to the injured site for the subsequent tissue regeneration. Therefore, utilization of endogenous cells will be an intense source to induce tissue regeneration. If endogenous cells can be accumulated to the injured site to be repaired by any method, it will be more realistic to promote the tissue regeneration based on the body cells.

Scaffold is a platform of three dimensions which allows cells to properly localize in the site necessary or proliferate and differentiate for their functional organization, and works as an artificial extracellular matrix (ECM) of body tissue. It is well known that the ECM is not only a physical support of cells, but also provides a local environment for cell proliferation, differentiation, or morphogenesis which contributes to organogenesis and tissue repairing [18]. Generally, in the body, cells survive and biologically function attaching and interacting with the ECM. Therefore, only when cells are transplanted directly, they cannot always survive and function without the presence of cell scaffold [19]. The cell scaffold is very important to allow cells to function *in vivo*. The properties required for the scaffold are shown in Table 1.

Little has been reported on cell scaffolds with all requirements at present. Different types of cell scaffolds have been investigated with biodegradable materials. As synthetic materials, poly(glycolic acid), poly(D,L-lactide), poly(L-lactide), polyethylene glycol, poly(ε-caprolactone), poly(dioxanone), their copolymers, and some ceramics are utilized for scaffold preparation [20-22]. The materials have various advantages, such as the easy property modification, well-controlled chemical composition, and processing capability. On the other hand, there are some drawbacks to be resolved. The foreign body reaction to the material, the toxicity of degradation products, and the poor biocompatibility, should be improved. Compared with the synthetic, the materials of collagens [23], fibrin [24], polysaccharides [25], and decellularized matrices [26] show low body reaction and good biocompatibility. It should be, however, noted that the difficulty in modification of material degradation or stiffness and the heterogeneity of chemical composition are in issue. Among the scaffold of natural material, a sponge

Table 1. Requirements of cell scaffold of artificial ECM

- Assistance of cell attachment, proliferation, and differentiation
- Space and place making for *in vivo* tissue regeneration
- Shape determination of tissue to be regenerated
- Mechanical support until to the accomplishment of tissue regeneration
- Structure maintenance for cells infiltration and vasculature
- Structure maintenance for oxygen and nutrients supplying to cells
- Structure maintenance for exclusion of metabolic wastes
- Degradability not to suppress the process of tissue regeneration.
- Substitution for other functions of natural ECM

form of type I collagen has been used clinically as an artificial dermis for about twenty years to prove the biocompatibility and biosafety [27]. The type I collagen is ubiquitously present in our body and one of the main components of ECM. Moreover, it has a cell-binding domain for the functional maintenance of various cells [28, 29]. In addition, other components of ECM, such as laminin and polysaccharides, are known to affect the cell behavior [30-32].

Bio-signaling molecules biologically maintain and regulate the activity of cells to migrate, proliferate, or differentiate in the tissue. They include growth factors, cytokines, hormones, and other bioactive substances. The bio-signaling molecules are generally unstable in the body and their *in vivo* half-life period is very short. Therefore, in the body, they are normally stabilized in a certain fashion. For example, the molecules are normally immobilized in the ECM of tissue for their storage and

stabilization. The molecules immobilized, when required, are detached from the ECM or released to function by the enzyme secreted from cells. This phenomenon is strictly regulated and takes place in a time- and concentration-dependent manner or in physiological and pathological events, such as inflammation [33] and wound-healing [34]. Therefore, only by the simple administration of molecule solution into the body, the *in vivo* function cannot always be expected. This is because the molecules administered rapidly diffuse from the site injected and digested or deactivated [35, 36]. As one possible way to breakthrough the issue, drug delivery system (DDS) is promising. The DDS technology enables the bio-signaling molecule to efficiently function *in vivo* and enhance the biological effect since it facilitates the molecule at the right place, the proper time, and right concentration [37]. For example, the controlled release of bio-signaling molecule at the site of action over an extended time period is achieved by incorporating the molecule into an appropriate carrier. Tabata *et al.* have developed the controlled release of biologically active bio-signaling molecules from hydrogels of gelatin [38, 39]. Gelatin is a biodegradable protein which is prepared through an acid and alkaline process of collagen. Gelatin has been extensively utilized for pharmaceutical and medical purposes, and the biosafety has been proven through the long clinical applications [40-42]. It has been demonstrated that various growth factors of bio-signaling molecule were released from gelatin hydrogels [38, 43] to experimentally confirm feasibility of the release system in bio-signaling molecules-induced tissue regeneration.

Adipose tissue is a soft tissue in our body and has many biological roles, such as the energy accumulation, lubricant between the skin and muscle, the construction of

body contour as breast tissue, and cytokine secretion [44, 45]. In addition, capillary and blood vessels play an important role in the body network to supply oxygen and nutrients to cells and tissues and exclude their wastes [46]. Bone tissue is one of the skeletal components to maintain the body shape and supporting loads, while it contains the bone marrow inside to generate and supply blood and other cells [47]. Autologous transplantation of adipose tissue grafts of a few millimeters size and semiliquid, has been clinically performed for depressed regions or scars in the breast and facial areas [48, 49]. However, these treatments meet some problems, such as the absorption and fibrosis of tissues grafted [50-53]. The main reason of the problems in the tissue transplantation is the shortage of blood flow in the grafted tissues. To breakthrough the problem, the regeneration of capillary and blood vessels would be required. In addition, the vascular regeneration is an important strategy to treat ischemic diseases. Recently, artificial bone substitutes prepared from ceramics or metals have widely been used after the excision of bone cancers clinically [54]. However, the substitution treatment does not always bring out good clinical results. This is because the substitution material is a foreign material for the living body. Therefore, if it is possible to artificially induce regeneration of autologous tissues at the defect site, this will be a promising substitute for the tissue transplantation and substitution by artificial materials.

There are two research strategies of tissue regeneration. The first strategy is to use cells that proliferate and differentiate for tissue regeneration. There have been many research reports for this approach. For example, regeneration of adipose tissue in the rat subcutis has been reported by use of porous scaffold of poly(lactic-*co*-glycolic acid) preseeded with autologously isolated preadipocytes [55, 56]. It is reported that the

regeneration of bone tissue was achieved by implantation of hydroxyapatite porous materials seeded with bone marrow cells [57]. The second way is to induce *in vivo* tissue regeneration based on the self-healing potentials of precursor or stem cells, such as preadipocytes, vascular endothelial cells, and osteoblasts which are originally present in the body. It is well known that sprouting and branching of capillary vessels is based on the mechanism of vasculogenesis [58]. The phenomenon is induced by precursor or stem cells which are originally present in the body. The cells around the injured site and circulating in the blood circulation are accumulated to the site to assist the natural process of tissue regeneration. Cell accumulation is promoted by the functions of cytokines [59] and chemokines [9, 12, 60]. If it is possible to provide a local environment suitable for the promoted cell accumulation and the subsequent proliferation and differentiation, tissue regeneration will be expected without the exogenous transplantation of cells. To create the local environment of cell-based tissue regeneration, the cell scaffold and DDS technologies of bio-signaling molecules have been investigated in this study. To achieve tissue regeneration at higher efficiency, various materials to combine between the two technologies are designed and prepared.

The objective of this thesis is to investigate the way how to induce *in vivo* regeneration of various tissues, such as adipose tissues, capillary vessels, and bone tissues, by using the cell scaffolds and controlled release materials of bio-signaling molecules with or without combination of precursor cells. PART I is concerned with the regeneration of adipose tissue by the collagen-based cell scaffolds and gelatin hydrogel microspheres for bFGF release combined with preadipocytes. The effect of the material properties, the scaffold degradability, and the time profile of bFGF release on the

adipose tissue regeneration is evaluated. PART II focuses on the regeneration of adipose tissues, capillary vessels, and bone tissues, by making use of cells originally present in the body. After the cell scaffolds and the hydrogel microspheres for bFGF release are combined and implanted, the process of *de novo* and *in situ* adipogenesis is evaluated. If the scaffold of artificial ECM is biologically compatible, it is highly expected that cells residing around the implanted scaffold infiltrate into it, and their natural proliferation and differentiation result in the *in situ* cell-based tissue regeneration. In addition, some bio-signaling molecules which enable circulating progenitor and stem cells to recruit and accumulate to the tissue site to be regenerated, are used. For example, it is experimentally demonstrated that stromal cell-derived factor (SDF)-1 of a chemokine functions as a cell attracting factor to promote the recruitment of body cells to the site where the SDF-1 is present, for cell-based tissue regeneration [61]. If the chemokine is used in a controlled release fashion, it is conceivable that an efficient recruitment of autologous key cells to the present site of chemokine is enhanced to achieve cell-based tissue regeneration. In this study, this new strategy where the cells inherently present in the body are actively attracted to the site to be regenerated for tissue regeneration, is designed and investigated to evaluate whether or not it functions well for tissue regeneration.

Chapter 1 is concerned with the regeneration of adipose tissue by collagen sponge scaffolds and gelatin microspheres containing bFGF combined with human preadipocytes. After subcutaneous implantation of the collagen sponges incorporating gelatin microspheres containing bFGF plus human preadipocytes, the regeneration of adipose tissue was histologically evaluated. Significant regeneration of adipose tissue

was observed only for the combination of all the three components. Maximum area of adipose tissue regenerated was histologically detected when the bFGF dose was 1μg/site and the seeded cell number was $10^{5/}$site. No adipogenesis was achieved at the implanted site of collagen sponge alone and that incorporating either gelatin microspheres containing bFGF or human preadipocytes as well as the mixed gelatin microspheres containing bFGF and human preadipocytes.

Chapter 2 describes the effect of material properties on the regeneration of adipose tissue by collagen sponge scaffolds and gelatin microspheres containing bFGF combined with human preadipocytes. *In vivo* regeneration of adipose tissue was histologically evaluated after implantation of human preadipocytes combining with collagen sponges with different biodegradabilities and gelatin microspheres containing bFGF which have different release profiles. The sponge biodegradability could be regulated by changing the crosslinking conditions in sponge preparation. The time profile of bFGF release could be changed by altering the biodegradability of gelatin microspheres used. The area of adipose tissue regenerated became maximum in the case of the collagen sponge with a moderate degradation rate. When the sponge is degraded fast, it cannot function as the scaffold of cell proliferation and the space provider for tissue regeneration. If the sponge is not degraded and remains for a long time period, the remaining will physically impair the process of tissue regeneration. The balanced biodegradability of sponge scaffold would enable preadipocytes to induce regeneration of adipose tissue. No influence of the bFGF release profile on the tissue area was observed.

Chapter 3 investigates the effect of scaffold chemical composition on the proliferation and differentiation of human preadipocytes. Preadipocytes were cultured on various substrates coated with type I collagen mixed with various ECM components, such as type IV collagen, laminin, hyaluronic acid, and gelatin. Proliferation and adipogenic differentiation of preadipocytes greatly depended on the type of substrates. By adding type IV collagen and laminin-1 to type I collagen, good proliferation and adipogenic differentiation of preadipocytes were observed.

PART II of this thesis is directed to investigate the regeneration of various tissues by the sponge scaffold and the release materials of bio-signaling molecules for tissue regeneration without cells transplantation. Chapter 4 describes the time course of *de novo* adipogenesis by Matrigel® of natural scaffold and gelatin microspheres containing bFGF. The Matrigel® is a basement membrane extract from mouse sarcoma and composed of basement membrane proteins, such as type IV collagen and laminin [62, 63]. When measured in the Matrigel® co-implanted with the gelatin microspheres containing bFGF, the number of cells infiltrated into Matrigel® increased to a significantly high extent compared with that of Matrigel® plus bFGF solution. The controlled release would enable bFGF to enhance the biological activities of promoted angiogenesis and preadipocytes proliferation, resulting in the enhanced regeneration of adipose tissue. Matrigel® alone was much less effective in inducing the regeneration of adipose tissue.

Chapter 5 is concerned with *in situ* adipogenesis by the collagen scaffold and the gelatin microspheres containing bFGF. After the collagen sponge scaffold was incorporated with the microspheres containing bFGF and implanted into a defect of rat

fat tissue, adipogenesis was histologically evaluated at the implanted site of scaffold. *In situ* regeneration of adipose tissue accompanied with angiogenesis was observed in the scaffold implanted with the microspheres containing bFGF with and without preadipocyte implantation. The regeneration extent was significantly higher than that induced by the collagen sponge plus free bFGF at the same dose. The controlled release would enable bFGF to enhance the infiltration of preadipocytes from the surrounding fat tissue to the defect and increase the cell number, in addition to bFGF-induced angiogenesis. It is possible that these bFGF effects consequently promote *in situ* regeneration of adipose tissue.

Chapter 6 proceeds to the preparation of gelatin-based hydrogels for the controlled release of SDF-1 and examines the SDF-1-induced cell accumulation and angiogenesis. Gelatin was chemically derivatized to give it different chemical properties of electric charge and hydrophobicity, while the feasibility as the material for the controlled release of SDF-1 was evaluated. Among the derivatives, succinylated gelatin of an anionic charge was the most suitable material to prepare hydrogels for SDF-1 release. The time profile of SDF-1 release from the hydrogel of succinylated gelatin could be controlled by changing the water content of hydrogel which can be modified with the conditions of hydrogel preparation. The time profile of SDF-1 release was in good accordance with that of hydrogel degradation. This indicates that SDF-1 can be released from the hydrogel as a result of hydrogel degradation. When evaluated after the subcutaneous implantation of succinylated gelatin hydrogels containing SDF-1 or administration of SDF-1 solution, significantly stronger angiogenesis by the hydrogel containing SDF-1 was observed. The hydrogel implantation enhanced the mRNA level

of SDF-1 receptor around the site implanted. The controlled release would enable SDF-1 to enhance the recruitment and accumulation of cells, resulting in the enhanced angiogenesis. This result clearly indicates that the release of SDF-1 with a cell accumulation activity can enhance the recruitment of angiogenic cells and consequently cell-based angiogenesis.

Chapter 7 is concerned with the preparation of gelatin microspheres for the controlled release of BMP-2 and the BMP-2-induced cells accumulation and bone regeneration. A gelatin hydrogel containing BMP-2 was implanted into the back subcutis of enhanced green fluorescent protein (GFP)-chimeric mice to induce the ectopic regeneration of bone tissue. The tissue around the hydrogel-implanted site was sampled at different time intervals, and the histological section was prepared to evaluate the number ratio of GFP- and osteocalcin-positive cells recruited into the bone tissue regenerated. The number of GFP-positive bone marrow-derived cells accumulated depended on the BMP-2 release profile. Significantly larger regeneration of bone tissue and the tissue maintenance were observed.

In summary, this thesis describes importance and necessity of environmental design by making use of cell scaffold and the controlled release system of bio-signaling molecules for tissue regeneration with or without the co-transplantation of cells. It is concluded that the appropriate combination of cell scaffold and local delivery of bio-signaling molecules is key to induce the *in vivo* tissue regeneration which can be achieved not only by the co-transplantation of precursor cells, but also by actively utilizing the key cells present in the body.

REFERENCES

1. Stocum DL. Regenerative Biology and Medicine. New York: Academic Press, 2006.
2. Langer R. Tissue Engineering: Perspectives, Challenges, and Future Directions. Tissue Eng 2007;13:1-2.
3. Langer R, Vacanti JP. Tissue Engineering. Science 1993;260:920-6.
4. Lanza R, Langer R, Vacanti JP. Principles of Tissue Engineering, 3rd Edition. New York: Academic Press, 2007.
5. Morrison SJ. Stem Cell Potential: Can Anything Make Anything? Curr Biol 2001;11:R7-9.
6. Svendsen CN, Smith AG. New Prospects for Human Stem-Cell Therapy in the Nervous System. Trends Neurosci 1999;22:357-64.
7. Levenberg S, Langer R. Advances in Tissue Engineering. Curr Top Dev Biol 2004;61:113-34.
8. Lavik E, Langer R. Tissue Engineering: Current State and Perspectives. Appl Microbiol Biotechnol 2004;65:1-8.
9. Hattori K, Heissig B, Rafii S. The Regulation of Hematopoietic Stem Cell and Progenitor Mobilization by Chemokine SDF-1. Leuk Lymphoma 2003;44:575-82.
10. Juarez J, Bendall L, Bradstock K. Chemokines and Their Receptors as Therapeutic Targets: The Role of the SDF-1/CXCR4 Axis. Curr Pharm Des 2004;10:1245-59.
11. Kollet O, Dar A, Lapidot T. The Multiple Roles of Osteoclasts in Host Defense: Bone Remodeling and Hematopoietic Stem Cell Mobilization. Annu Rev Immunol 2006;25:51-69.

12. Lapidot T, Dar A, Kollet O. How Do Stem Cells Find Their Way Home? Blood 2005;106:1901-10.
13. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The Pleiotropic Effects of the SDF-1-CXCR4 Axis in Organogenesis, Regeneration and Tumorigenesis. Leukemia 2006;20:1915-24.
14. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the Stromal Cell-Derived Factor-1/CXCR4 Pathway in Prostate Cancer Metastasis to Bone. Cancer Res 2002;62:1832-7.
15. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating Osteoblast-Lineage Cells in Humans. N Engl J Med 2005;352:1959-66.
16. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating Skeletal Stem Cells. J Cell Biol 2001;153:1133-40.
17. Wan C, He Q, Li G. Allogenic Peripheral Blood Derived Mesenchymal Stem Cells (MSCs) Enhance Bone Regeneration in Rabbit Ulna Critical-Sized Bone Defect Model. J Orthop Res 2006;24:610-8.
18. Morrison SJ, Spradling AC. Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance Throughout Life. Cell 2008;132:598-611.
19. Chiarugi P, Giannoni E. Anoikis: A Necessary Death Program for Anchorage-Dependent Cells. Biochem Pharmacol 2008;76:1352-64.
20. Holland TA, Mikos AG. Biodegradable Polymeric Scaffolds. Improvements in Bone Tissue Engineering through Controlled Drug Delivery. Adv Biochem Eng Biotechnol 2006;102:161-85.
21. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and Bioactive

Porous Polymer/Inorganic Composite Scaffolds for Bone Tissue Engineering. Biomaterials 2006;27:3413-31.

22. Yang S, Leong KF, Du Z, Chua CK. The Design of Scaffolds for Use in Tissue Engineering. Part I. Traditional Factors. Tissue Eng 2001;7:679-89.
23. Yannas IV. Similarities and Differences between Induced Organ Regeneration in Adults and Early Foetal Regeneration. J R Soc Interface 2005;2:403-17.
24. Bensaid W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H. A Biodegradable Fibrin Scaffold for Mesenchymal Stem Cell Transplantation. Biomaterials 2003;24:2497-502.
25. Mano JF, Silva GA, Azevedo HS, Malafaya PB, Sousa RA, Silva SS, Boesel LF, Oliveira JM, Santos TC, Marques AP, Neves NM, Reis RL. Natural Origin Biodegradable Systems in Tissue Engineering and Regenerative Medicine: Present Status and Some Moving Trends. Journal of The Royal Society Interface 2007;4:999-1030.
26. Badylak SF. The Extracellular Matrix as a Biologic Scaffold Material. Biomaterials 2007;28:3587-93.
27. Yannas IV, Burke JF. Design of an Artificial Skin. I. Basic Design Principles. J Biomed Mater Res 1980;14:65-81.
28. Cen L, Liu W, Cui L, Zhang W, Cao Y. Collagen Tissue Engineering: Development of Novel Biomaterials and Applications. Pediatr Res 2008;63:492-6.
29. Glowacki J, Mizuno S. Collagen Scaffolds for Tissue Engineering. Biopolymers 2008;89:338-44.
30. Takagi J. Structural Basis for Ligand Recognition by Integrins. Curr Opin Cell Biol

2007;19:557-64.

31. Zhao YY, Takahashi M, Gu JG, Miyoshi E, Matsumoto A, Kitazume S, Taniguchi N. Functional Roles of N-Glycans in Cell Signaling and Cell Adhesion in Cancer. Cancer science 2008;99:1304-10.
32. Gu J, Taniguchi N. Regulation of Integrin Functions by N-Glycans. Glycoconj J 2004;21:9-15.
33. Luster AD, Alon R, von Andrian UH. Immune Cell Migration in Inflammation: Present and Future Therapeutic Targets. Nat Immunol 2005;6:1182-90.
34. Monaco JL, Lawrence WT. Acute Wound Healing an Overview. Clin Plast Surg 2003;30:1-12.
35. Manning MC, Patel K, Borchardt RT. Stability of Protein Pharmaceuticals. Pharm Res 1989;6:903-18.
36. Wang YJ, Hanson A. Perenteral Formulations of Proteins and Peptides: Stability and Stabilizers. J Parenter Sci Technol 1988;42:S2-S26.
37. Sokolsky-Papkov M, Agashi K, Olaye A, Shakesheff K, Domb AJ. Polymer Carriers for Drug Delivery in Tissue Engineering. Adv Drug Deliv Rev 2007;59:187-206.
38. Ikada Y, Tabata Y. Protein Release from Gelatin Matrices. Adv Drug Deliv Rev 1998;31:287-301.
39. Kushibiki T, Tabata Y. A New Gene Delivery System Based on Controlled Release Technology. Curr Drug Deliv 2004;1:153-63.
40. Heene DL, Zekorn D, Lasch HG. Gelatine Plasma Volume Expanders: Chemistry, Biological Activities and Clinical Experiences. Bibl Haematol 1968;29:907-13.
41. Marois Y, Chakfe N, Deng X, Marois M, How T, King MW, Guidoin R.

Carbodiimide Cross-Linked Gelatin: A New Coating for Porous Polyester Arterial Prostheses. Biomaterials 1995;16:1131-9.

42. Zekorn D. Intravascular Retention, Dispersal, Excretion and Break-Down of Gelatin Plasma Substitutes. Bibl Haematol 1969;33:131-40.
43. Yamamoto M, Takahashi Y, Tabata Y. Controlled Release by Biodegradable Hydrogels Enhances the Ectopic Bone Formation of Bone Morphogenetic Protein. Biomaterials 2003;24:4375-83.
44. MacLaren R, Cui W, Cianflone K. Adipokines and the Immune System: An Adipocentric View. Adv Exp Med Biol 2008;632:1-21.
45. Fruhbeck G. Overview of Adipose Tissue and Its Role in Obesity and Metabolic Disorders. Methods Mol Biol 2008;456:1-22.
46. Guyton AC, Hall JE. Textbook of Medical Physiology 11st Ed. Oxford, UK: Elsevier, 2005.
47. Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of Bone Biology. New York: Academic Press, 1996.
48. Ellenbogen R. Free Autogenous Pearl Fat Grafts in the Face--a Preliminary Report of a Rediscovered Technique. Ann Plast Surg 1986;16:179-94.
49. Hartrampf CR, Scheflan M, Black PW. Breast Reconstruction with a Transverse Abdominal Island Flap. Plast Reconstr Surg 1982;69:216-25.
50. Smahel J. Failure of Adipose Tissue to Heal in the Capsule Preformed by a Silicone Implant. Eur J Plast Surg 1985;8:109-15.
51. Ersek RA. Transplantation of Purified Autologous Fat: A 3-Year Follow-up Is Disappointing. Plast Reconstr Surg 1991;87:219-27; discussion 28.

52. Fagrell D, Enestrom S, Berggren A, Kniola B. Fat Cylinder Transplantation: An Experimental Comparative Study of Three Different Kinds of Fat Transplants. Plast Reconstr Surg 1996;98:90-6; discussion 7-8.
53. Smahel J. Experimental Implantation of Adipose Tissue Fragments. Br J Plast Surg 1989;42:207-11.
54. Kao ST, Scott DD. A Review of Bone Substitutes. Oral Maxillofac Surg Clin North Am 2007;19:513-21, vi.
55. Patrick CW, Jr. Adipose Tissue Engineering: The Future of Breast and Soft Tissue Reconstruction Following Tumor Resection. Semin Surg Oncol 2000;19:302-11.
56. Patrick CW, Jr., Chauvin PB, Hobley J, Reece GP. Preadipocyte Seeded PLGA Scaffolds for Adipose Tissue Engineering. Tissue Eng 1999;5:139-51.
57. Ohgushi H, Caplan AI. Stem Cell Technology and Bioceramics: From Cell to Gene Engineering. J Biomed Mater Res 1999;48:913-27.
58. Czirok A, Zamir EA, Szabo A, Little CD. Multicellular Sprouting During Vasculogenesis. Curr Top Dev Biol 2008;81:269-89.
59. Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De Novo Adipogenesis in Mice at the Site of Injection of Basement Membrane and Basic Fibroblast Growth Factor. Proc Natl Acad Sci U S A 1998;95:1062-6.
60. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating Bone Marrow-Derived Osteoblast Progenitor Cells Are Recruited to the Bone-Forming Site by the CXCR4/Stromal Cell-Derived Factor-1 Pathway. Stem Cells 2008;26:223-34.
61. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A,

Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of Stromal-Cell-Derived Factor 1 on Stem-Cell Homing and Tissue Regeneration in Ischaemic Cardiomyopathy. Lancet 2003;362:697-703.

62. Baatout S, Cheta N. Matrigel: A Useful Tool to Study Endothelial Differentiation. Rom J Intern Med 1996;34:263-9.
63. Kleinman HK, Martin GR. Matrigel: Basement Membrane Matrix with Biological Activity. Semin Cancer Biol 2005;15:378-86.

PART I

REGENERATION OF ADIPOSE TISSUE BY CELL SCAFFOLD AND RELEASE MATERIALS OF BIO-SIGNALING MOLECULE COMBINED WITH CELLS

Chapter 1

Regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor combined with human preadipocytes

INTRODUCTION

In plastic and reconstructive surgery for augmentation of lost soft tissues [1], autologous transplantation of fat grafts of a few millimeters size and semiliquid has been clinically performed for depressed regions or scars in the breast and facial areas [2, 3]. However, this treatment often meets some problems, such as the absorption and fibrosis of tissues grafted [4-7]. Thus, if it is possible to artificially induce regeneration of adipose tissue at such a defect site, the regeneration will be a promising substitute for the tissue graft.

Recently, tissue engineering has been being noticed as a newly emerging biomedical technology to repair or regenerate a body defect by combining cells of high proliferation and differentiation potential with an artificial matrix of cell scaffold and growth factor [8]. This tissue engineering technology is also applicable for regeneration of adipose tissue, and some trials have been reported on adipose tissue engineering [9-12]. There are two possible strategies based on tissue engineering to induce

regeneration of adipose tissue. The first strategy is to make use of cells that are potential for proliferation and differentiation to regenerate adipose tissue. The cells are brought into a body site where regeneration of adipose tissues is expected. For example, it is reported that a preadipocyte cell line induced regeneration of adipose tissue at the site of subcutaneous injection to nude mice [13]. Patrick *et al.* have succeeded in regenerating adipose tissue in the rat subcutis by use of porous scaffold of poly(lactic-co-glycolic acid) pre-seeded with autologously isolated preadipocytes [10, 14]. Adipose tissue engineering by use of collagen scaffold combined with human preadipocytes has been reported [15, 16]. The second way is to induce *in vivo* regeneration of adipose tissue based on precursor or stem cells, like preadipocytes, originally existing in the body. If it is possible to provide a local environment suitable for the proliferation and differentiation of such cells, regeneration of adipose tissue will be expected without exogenous transplantation of cells necessary for adipogenesis.

This chapter was undertaken to examine whether or not a collagen sponge functions as the scaffold for regeneration of adipose tissue based on gelatin microspheres for bFGF release. Preadipocytes, a precursor of adipocytes, were prepared from human fat tissue isolated in breast surgery. Following subcutaneous implantation of collagen sponge incorporating human preadipocytes and the gelatin microspheres containing bFGF into the back of nude mice, regeneration of adipose tissue was evaluated from the viewpoint of histological area occupied by adipose tissue regenerated and compared with that of collagen sponge incorporating with preadipocytes plus bFGF in the solution form. The effect of the number of preadipocytes transplanted and the bFGF dose on the adipogenesis was examined.

EXPERIMENTAL

Materials

An aqueous solution of human recombinant bFGF with an isoelectric point (IEP) of 9.6 (10 mg/ml) was kindly supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. A gelatin sample with an IEP of 5.0 (Nitta Gelatin Co., Osaka, Japan) was prepared through an alkaline process of type I collagen obtained from bovine bone. The freeze-dried sponge sheet (80 x 60 mm^2, 3 mm thickness) of porcine tendon type I collagen was kindly supplied by Gunze Co., Ltd., Kyoto, Japan. The sponge sheet was prepared by the dehydrothermal (140 °C, 6 hr) and the subsequent glutaraldehyde (GA) crosslinking (0.2 %(w/v), 4 °C, 12 hr) of collagen. The porosity and pore size of sponge were 90 % and 60-100 μm. The collagen sheet was cut by scissors to prepare square sheets (5 x 5 mm^2) for *in vivo* experiments. GA, glycine, and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan and used without further purification.

Preparation of gelatin microspheres containing bFGF

Gelatin microspheres were prepared through GA crosslinking of gelatin aqueous solution in an emulsion state. Immediately after mixing of 25 %(w/v) GA aqueous solution (25 μl) with 10 ml of 10 %(w/v) gelatin aqueous solution preheated at 40 °C, the mixed aqueous solution was dropwise added to 375 ml of olive oil under stirring at 420 rpm and 40 °C to obtain a W/O emulsion. Stirring was continued for 24 hr at 25 °C to allow the gelatin to be chemically crosslinked. After addition of 100 ml of

acetone to the reaction mixture, the resulting microspheres were collected by centrifugation (4 °C, 3,000 rpm, 5 min) and washed five times with acetone by centrifugation. The washed microspheres were placed in 100 ml of 100 mM glycine aqueous solution containing Tween 80 [0.1 %(w/v)], followed by agitation at 37 °C for 1 hr to block the residual aldehyde groups of unreacted GA. Then, the crosslinked microspheres were washed twice with double-distilled water (DDW) by centrifugation, freeze-dried, and sterilized with ethylene oxide gas. The water content of the gelatin microspheres was 95 %, when calculated from the microsphere volume before and after swelling in PBS for 24 hr at 37 °C. The microsphere diameter was measured by viewing at least 100 microspheres with a light microscope and found to range from 60 to 130 μm in the state of PBS swelling.

The original bFGF solution was diluted with DDW to adjust the bFGF concentrations at 10, 20, 100, 200, and 1000 μg/ml. The aqueous solution of bFGF (50 μl) was dropped onto 2 mg of freeze-dried gelatin microspheres, followed by leaving at 25 °C for 3 hr for impregnation of bFGF into the microspheres. The bFGF solution was completely absorbed into the microspheres through the impregnation process because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres.

A series of study [17-21] indicated that bFGF was released from the gelatin hydrogel microspheres of release carrier not by simple diffusion, but by the water-solubilization of bFGF accompanied with hydrogel degradation. bFGF is immobilized into the hydrogel microspheres based on the electrostatic complexation between the basic bFGF and acidic gelatin molecules. The complexed bFGF is not

released from the hydrogels unless they are degraded *in vivo* to form water-soluble gelatin fragments. Animal experiments revealed that the time profile of *in vivo* retention was in good accordance with that of *in vivo* hydrogel degradation [21]. When evaluated in terms of angiogenesis, the biological activity of bFGF released from gelatin hydrogels could be detected and lasted for longer time periods as the degradation time period of hydrogels was prolonged [22]. The gelatin microspheres containing bFGF used here were degraded with time in the back subcutis of mice to completely disappear 3 weeks later. Based on the mechanism of bFGF release from this hydrogel system, the gelatin microspheres achieve the controlled release of biologically active bFGF over 3 weeks.

Isolation and culture of human preadipocytes

Human preadipocytes were primarily isolated from human adipose tissues that were obtained in the reduction mammaplastic surgery of breast cancer patients with informed-consent at Kyoto University Hospital. The adipose tissue was washed with phosphate-buffered saline solution (PBS, pH 7.4) to carefully remove blood cells, then minced, and digested by 520 U/ml collagenase (Nitta Gelatin, Osaka, Japan) in a water bath at 37 °C for 60 min under shaking. The digested was suspended in Medium 199 containing 10 vol% fetal bovine serum (FBS), followed by centrifugation (200 x g, 5 min at 4 °C) to remove the supernatant. After washing twice with the medium, the cells obtained were cultured in a cell culture flask (75 cm^2, CORNING 430720, 1 x 10^3 cells/cm^2) in the medium containing 0.1 μg/ml of bFGF at 37 °C and 5 % CO_2 - 95 % air atmosphere pressure. The cells were expanded without subculturing and subjected to

in vivo experiments. The cell morphology was fibroblast-like. When cultured in the presence of 50 nM of insulin, 100 nM of dexamethasone, 10 μg/ml of transferrin, and 200 pM of triiodethyronine for 14 days, the cells accumulated lipid droplets inside. This suggests that the cells isolated had an inherent nature to differentiate into mature adipocytes.

***In vivo* experiments**

The gelatin microspheres (2 mg) swollen with the bFGF aqueous solution were mixed with the suspension of human preadipocytes at cell densities of 2 x 10^4, 5 x 10^4, 1 x 10^5, and 5 x 10^5 cells/50 μl culture medium. As control, 10 μl of aqueous solutions containing 1 μg of bFGF was similarly mixed with 1 x 10^5 cells of human preadipocytes. The mixed suspension was dropped on the freeze-dried collagen sponge sheet (5 x 5 mm^2, 3 mm thickness) for the impregnation, followed by incubation for 3 hr under the same conditions as described above for the collagen sponge and gelatin microspheres containing bFGF combined with human preadipocytes.

Under anesthesia, the collagen sponge and gelatin microspheres containing bFGF combined with human preadipocytes was implanted into the back subcutis of female BALB/c nude mice, 6 weeks of age (Shimizu Laboratory Supply, Kyoto, Japan), 1.5 cm distance away from the tail root at the body center. As controls, mice received implantation of the collagen sponge incorporating human preadipocytes and free bFGF, or the mixture of two components selected from the sponge, the microspheres containing bFGF, and human preadipocytes. All animal experiments were performed according to the Guidelines of Animal Experiment of Kyoto University (1988). Each

experimental group was composed of six mice.

The mice, 6 weeks after implantation, were sacrificed by an overdose injection of anesthetic and the skin including the implanted site (2 x 2 cm^2) was carefully taken off for the subsequent biological examinations. The adipose tissues at the implanted site were assessed in terms of histological examination. The skin specimen was fixed with 10% neutralized formalin solution, embedded in paraffin, and sectioned (4 μm in thickness) at the portion of site implanted as central as possible, followed by staining with hematoxylin and eosin (HE). Microphotographs of six cross-sections from six different mice were taken at a similar magnification to histologically evaluate the regeneration of adipose tissue and angiogenesis. The same area of interest (three portions/cross-section, 0.8 x 0.5 mm^2) was randomly selected and the area occupied by matured adipocytes at the implanted site for every portion was measured by a computer program of NIH image analysis to express as the area of adipose tissue.

Immunohistological evaluation of adipose tissue regenerated by collagen scaffolds and gelatin microspheres containing bFGF combined with human preadipocytes

Deparaffined cross-sections of 4 μm thickness were rehydrated with PBS and incubated with mouse anti-human vimentin antibody (1:20 dilution) in a moist chamber for 24 hr at 4 °C. Then, the sections were rinsed with PBS three times and incubated with a second antibody-biotin conjugated rabbit anti-mouse IgG+IgA+IgM antibody (Histofine SAB-PO(M) kit, Nichirei Co., Tokyo, Japan) for 10 min at room temperature. After washing with PBS three times, the sections were incubated with peroxidase-conjugated streptoavidin solution (Histofine) for 5 min at room temperature.

Following extensive washing, the sections were exposed to peroxidase substrate DAB (3,3'-diaminobenzidine, Sigma) for 5 min at room temperature, then rinsed and counter-stained with hematoxylin. The section was viewed to assess the human-specific cellularity in the tissue.

Statistical analysis

All the data were analyzed by Fisher's LSD test for multiple comparison and the statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the mean + standard deviation of the mean (SD).

RESULTS

Regeneration of adipose tissue and vascularization by collagen scaffolds and gelatin microspheres containing bFGF combined with human preadipocytes

Figure 1 shows the tissue appearance of mouse subcutis 6 weeks after implantation of collagen sponge and gelatin microspheres containing bFGF or other agents combined with human preadipocytes. When the collagen sponge was implanted being incorporated with gelatin microspheres containing bFGF and preadipocytes, regeneration of tissue mass was found at the site implanted, while many blood vessels were distributed in the tissue formed. The similar change in tissue appearance was observed at the collagen sponge incorporating preadipocytes and free bFGF although the regeneration of blood vessels was less. Upon implanting with the collagen sponge

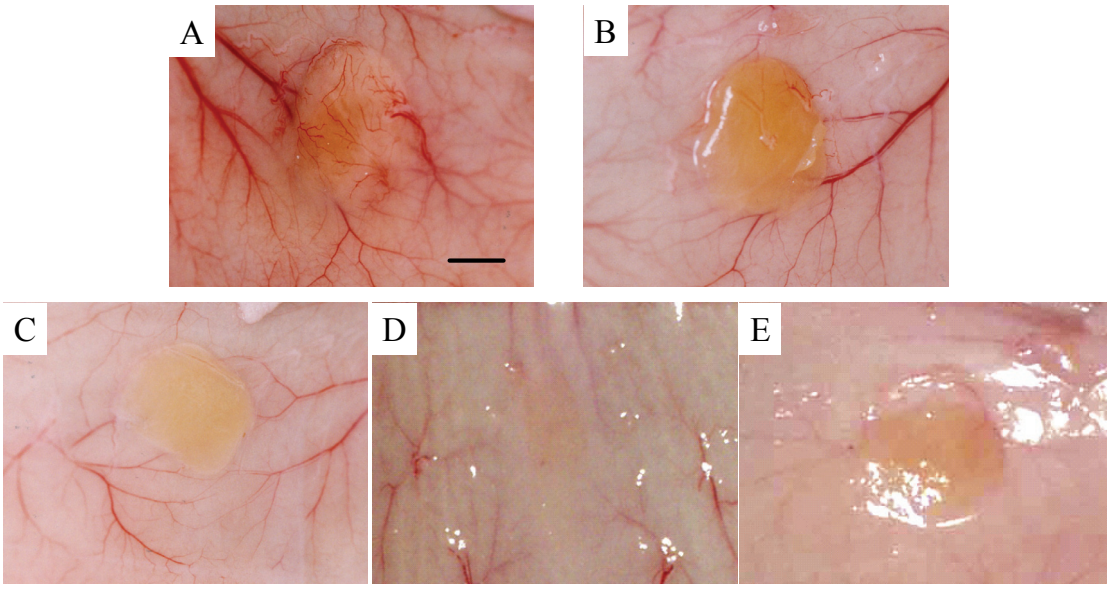

Figure 1. Tissue appearance of mouse subcutis 6 weeks after implantation of a collagen sponge incorporating human preadipocytes and gelatin microspheres containing 1 μg of bFGF (A), a collagen sponge incorporating human preadipocytes and 10 μg of free bFGF (B), a collagen sponge incorporating human preadipocytes (C), a mixture of human preadipocytes and gelatin microspheres containing 1 μg of bFGF (D), and a collagen sponge incorporating gelatin microspheres containing 1 μg of bFGF (E). Scale bar = 3mm. The number of preadipocytes transplanted is 1 x 10^5 cells/site.

incorporating either gelatin microspheres containing bFGF or preadipocytes and the mixture of preadipocytes and gelatin microspheres containing bFGF, such a change of tissue appearance was not observed at the implanted site.

Figure 2 shows the histological sections of the implanted site 6 weeks after implantation. Matured adipocytes accumulating lipid inside were observed in the tissue mass formed 6 weeks after implantation of collagen sponge incorporating human preadipocytes and gelatin microspheres containing bFGF, whereas the sponge incorporating human preadipocytes and free bFGF was less effective. For every

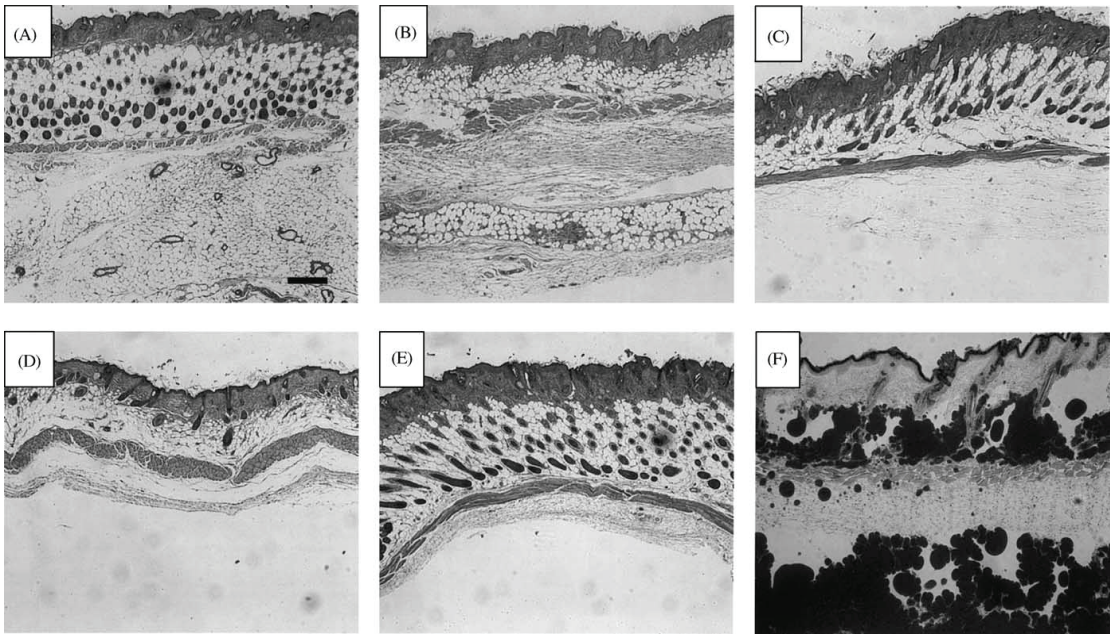

Figure 2. Regeneration of adipose tissue in the mouse subcutis 6 weeks after implantation of a collagen sponge incorporating human preadipocytes and gelatin microspheres containing 1 μg of bFGF (A), a collagen sponge incorporating human preadipocytes and 10 μg of free bFGF (B), a collagen sponge incorporating human preadipocytes (C), a mixture of human preadipocytes and gelatin microspheres containing 1 μg of bFGF (D), and a collagen sponge incorporating gelatin microspheres containing 1 μg of bFGF (E) (HE staining). (F) A Sudan III-stained section of group (A). The gelatin microspheres containing bFGF were completely degraded to disappear from the injected site. Scale bar = 300 μm. The number of preadipocytes transplanted is 1 x 10^5 cells/site.

combination of two from three components, the collagen sponge, gelatin microspheres containing bFGF, and human preadipocytes, no regeneration of adipose tissue was observed. Figure 3 shows the area of adipose tissue newly regenerated in the mouse subcutis 6 weeks after implantation. The area of adipose tissue regenerated was significantly large by the implantation of the collagen sponge incorporating the combination of human preadipocytes with gelatin microspheres containing bFGF

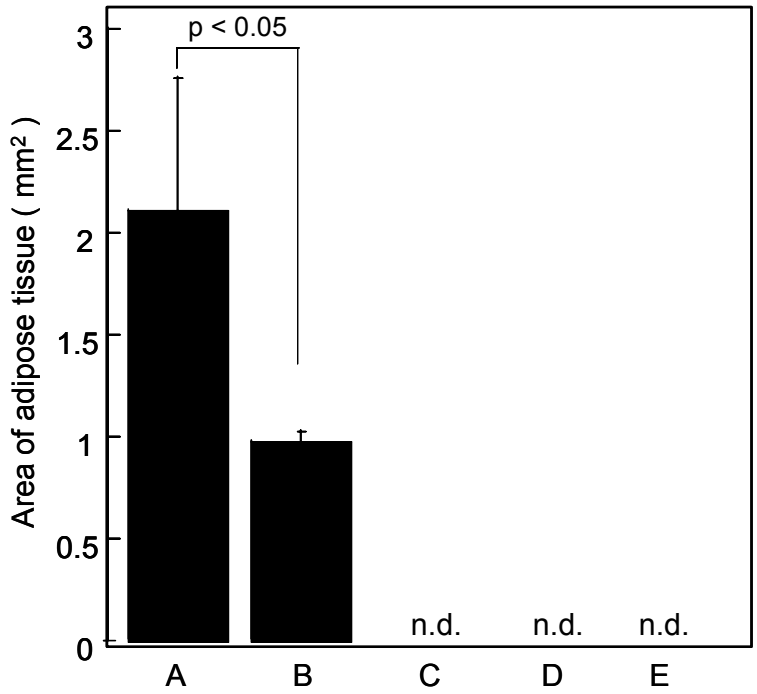

Figure 3. The area of adipose tissue regenerated at the implanted site of mouse subcutis 6 weeks after implantation of a collagen sponge incorporating 1 x 10^5 human preadipocytes and gelatin microspheres containing 1 μg of bFGF (A), a collagen sponge incorporating human preadipocytes and 10 μg of free bFGF (B), a collagen sponge incorporating gelatin microspheres containing 1 μg of bFGF (C), a collagen sponge incorporating human preadipocytes (D), and a mixture of human preadipocytes and gelatin microspheres containing 1 μg of bFGF (E). (n.d.: not detected)

compared with that of the combination with free bFGF. On the contrary, for other control groups, the area of adipose tissue was not detected.

Influence of the number of human preadipocytes transplanted and the bFGF dose on the *de novo* regeneration of adipose tissue

Figure 4 shows the influence of the preadipocytes number on the area of

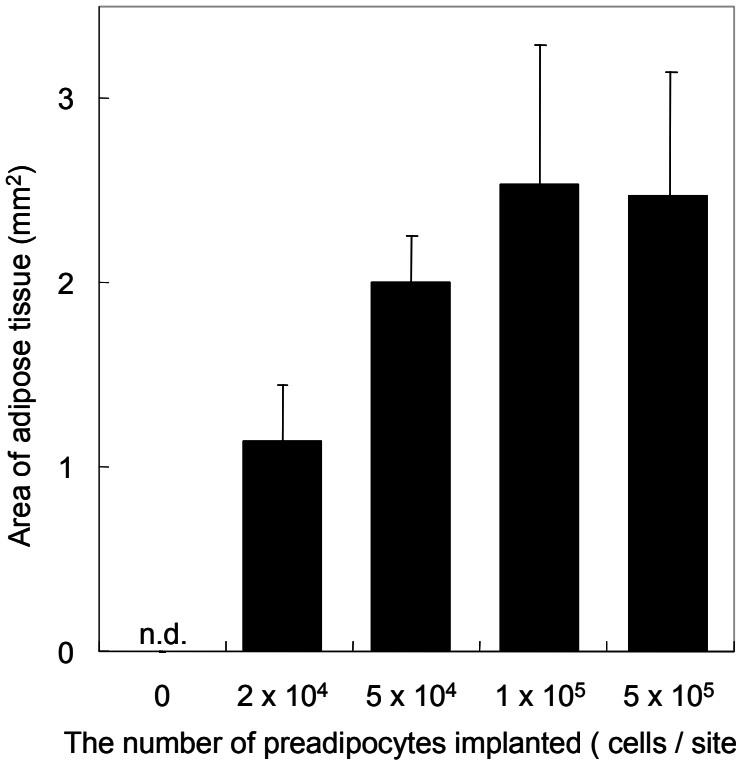

Figure 4. Effect of preadipocytes number on the area of adipose tissue regenerated 6 weeks after subcutaneous implantation of collagen sponge incorporating human preadipocytes and gelatin microspheres containing 1 μg of bFGF into the back of mice. (n.d.: not detected)

adipose tissue regenerated 6 weeks after implantation of the collagen sponge, human preadipocytes, and gelatin microspheres containing bFGF. The area increased with an increase in the number of human preadipocytes transplanted up to 1 x 10^5 cells/site and thereafter leveled off.

Figure 5 shows the histological sections of the implanted site 6 weeks after implantation of collagen sponges incorporating human preadipocytes and gelatin microspheres containing different doses of bFGF. Irrespective of the bFGF dose, the regeneration of adipose tissue was observed by use of gelatin microspheres

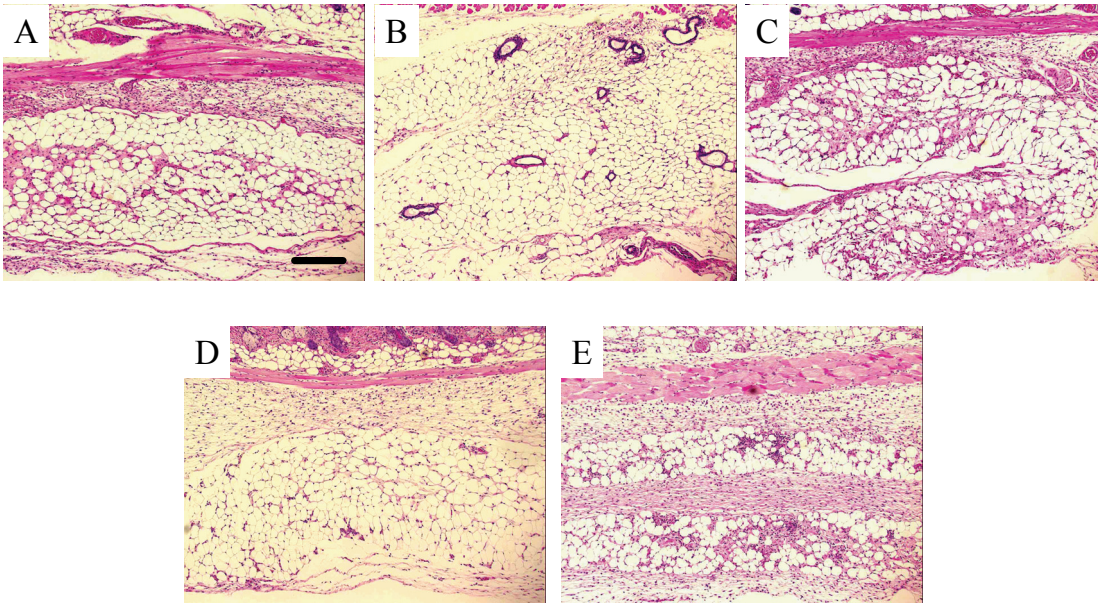

Figure 5. Effect of the bFGF dose on the *de novo* adipogenesis 6 weeks after subcutaneous implantation of collagen sponge incorporating human preadipocytes and gelatin microspheres containing bFGF into the back of mice: (A) 0.5, (B) 1, (C) 5, (D) 10, and (E) 50 μg of bFGF/site (1 x 10^5 preadipocytes/site) （HE staining, Scale bar = 200 μm）

containing bFGF. Among them, the maximum adipogenesis was achieved at the bFGF dose of 1 μg. Figure 6 shows effect of the bFGF dose on the area of adipose tissue regenerated 6 weeks after implantation. The area formed was significantly larger at the implanted site of collagen sponge scaffold incorporating human preadipocytes and gelatin microspheres containing 1μg of bFGF than that of other bFGF doses. The co-implantation of gelatin microspheres containing 50 μg of bFGF induced inflammatory reaction in and around the site implanted.

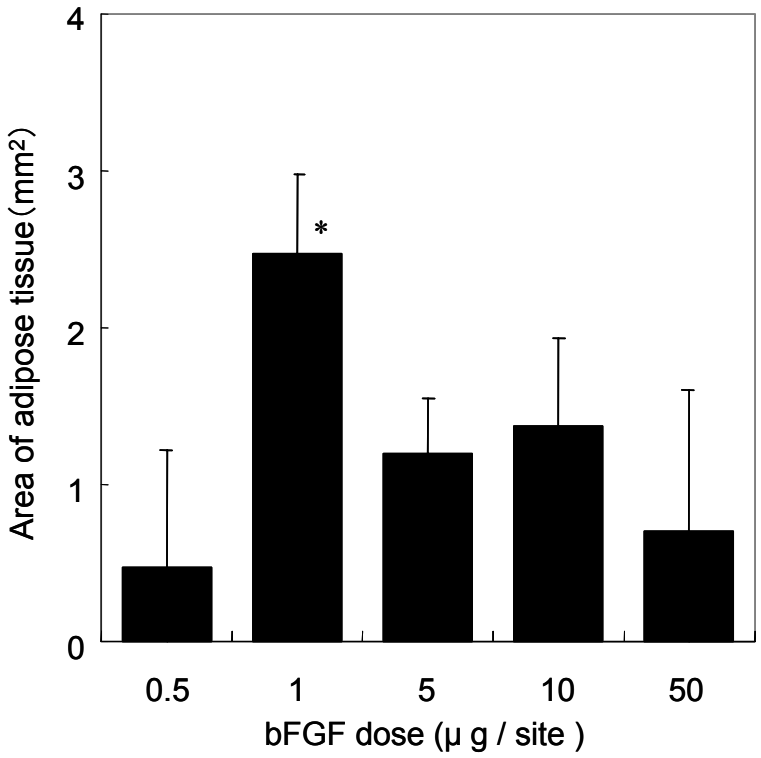

Figure 6. Effect of the bFGF dose on the area of adipose tissue regenerated 6 weeks after subcutaneous implantation of collagen sponge incorporating human preadipocytes and gelatin microspheres containing 1 μg of bFGF into the back of mice (1 x 10^5 preadipocytes/site). *: $p < 0.05$ significant against the area of adipose tissue of other groups.

Immunohistology of adipose tissue regenerated by a collagen sponge incorporating human preadipocytes and gelatin microspheres containing bFGF

Figure 7 shows the immunohistological section of adipose tissue regenerated by a collagen sponge incorporating human preadipocytes and gelatin microspheres containing bFGF. Apparently, the adipose tissue regenerated was throughout stained by the anti-human vimentin antibody, in contrast to the surrounding adipose tissues.

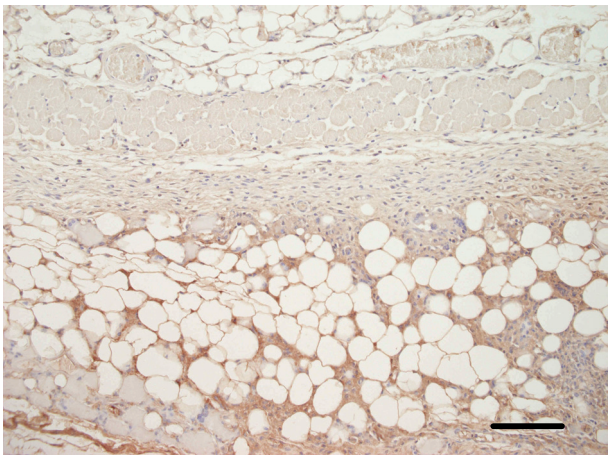

Figure 7. Immunohistological section of adipose tissue regenerated 6 weeks after implantation of a collagen sponge incorporating human preadipocytes and gelatin microspheres containing 1 μg of bFGF (1 x 10^5 preadipocytes/site) (Scale bar = 100 μm)

DISCUSSION

The hyperplastic formation of adipose tissue in aged animals by feeding with a high carbohydrate or high fat diet has intensively been investigated. It has been recognized in the recent cell biology that adipocyte linkage derives from multipotential mesenchymal stem cells with differentiation capacity [23]. The stem cells are morphologically and biochemically converted to matured adipocytes by way of adipose precursor cells [24, 25]. Among the precursor cells are preadipocytes that have committed or determined to become adipocytes and are included in interstitial cells having fibroblast-like morphology [26]. In addition, it has been demonstrated that the proliferation and differentiation of the precursor cells can be promoted depending on the microenvironment [13, 27]. It is well recognized that the number of adipocytes and their

precursor cells is only less than half that of total cells present in the adipose tissue and the remaining cells are vascular-related cells, like various blood cells, endothelial cells, and pericytes [12]. This tissue cellularity indicates that development of a vascular supply is essential for the generation and maintenance of adipose tissue. What is the local environment that allows adipose precursor cells to proliferate and differentiate into matured adipocytes? The present study clearly indicates that such an environment can be provided by implantation of the collagen sponge together with the release system of bFGF. There will be several reasons to be considered for the bFGF effect on induced adipogenesis. First, it is possible that the controlled release of bFGF induced angiogenesis, resulting in efficient proliferation and maturation of adipose precursor cells migrated in the in advance angiogenesis-induced scaffold because of good supply of oxygen and nutrients to the cells. Indeed, Tabata *et al.* indicated that such an angiogenesis-induced environment for tissue regeneration could be artificially created by implantation of Matrigel together with the release system of bFGF [28]. bFGF itself acts on the preadipocytes to accelerate their proliferation or other growth factors which are given by the bFGF-induced vasculature enable the cells to proliferate. It is conceivable that the collagen sponge plus the preadipocytes without the bFGF release system did not induce angiogenesis enough to maintain the survival of cells transplanted, resulting in no regeneration of adipose tissue. The collagen sponge does not have a function as the carrier of bFGF release [29], which will cause poor angiogenesis in the collagen sponge combined with preadipocytes and free bFGF. As a result, it is possible that the scaffold-cell-free bFGF combination results in poor adipogenesis compared with the scaffold-cell-released bFGF one. Secondly, we cannot rule out the possibility

that bFGF has a direct adipogenic effect. Sheep preadipocytes have been reported to differentiate in a culture medium containing bFGF [30]. It is conceivable that the controlled release of bFGF increases the number of preadipocytes and the rate of adipocyte differentiation, resulting in totally enhanced adipogenesis.

One promising way to effectively induce *in vivo* angiogenesis is to achieve the controlled release of bFGF over an extended period of time. Tabata *et al.* have demonstrated that significant angiogenesis was induced through the controlled release of biologically active bFGF from gelatin hydrogel microspheres, in marked contrast to bFGF administered in the solution form [17-20]. Histological observation revealed that co-implantation of the gelatin microspheres containing bFGF-induced angiogenesis in the collagen scaffold to a greater extent than that of free bFGF. It can be fairly certain that such promoted angiogenesis was one of the key contributing factors to significantly pronounced regeneration of adipose tissue. In addition, the adipogenic effect of bFGF should be considered. The bFGF dose dependence indicates that there is an optimal concentration range of bFGF for adipogenesis. Probably, a low dose of bFGF is not enough to exert its angiogenic or adipogenic effect even though the bioactive bFGF is released from the gelatin microspheres. On the other hand, when the bFGF dose is too high, in addition to the two effects of bFGF, the activity to accelerate infiltration of fibrous tissues into the collagen scaffold would become pronounced. A high dose of bFGF caused the inflammatory response at the collagen sponge implanted (Figure 5). It is possible that the inflammation occurrence is so severe that prevents the tissue from tissue regeneration.

Figure 7 clearly indicates that the adipose tissue regenerated throughout the

inside of implanted collagen was prepared based on human-derived cells. The tissue engineering strategy based on combination of cells, the scaffold, and growth factor was effective in inducing the regeneration of adipose tissue. However, the size of tissue formed was still too small to apply this technology to human therapy. Investigation about the scale-up of preadipocytes culture and technological design of enlarged tissue regeneration is underway at present.

REFERENCES

1. Billings E, Jr., May JW, Jr. Historical Review and Present Status of Free Fat Graft Autotransplantation in Plastic and Reconstructive Surgery. Plast Reconstr Surg 1989;83:368-81.
2. Ellenbogen R. Free Autogenous Pearl Fat Grafts in the Face--a Preliminary Report of a Rediscovered Technique. Ann Plast Surg 1986;16:179-94.
3. Hartrampf CR, Scheflan M, Black PW. Breast Reconstruction with a Transverse Abdominal Island Flap. Plast Reconstr Surg 1982;69:216-25.
4. Ersek RA. Transplantation of Purified Autologous Fat: A 3-Year Follow-up Is Disappointing. Plast Reconstr Surg 1991;87:219-27; discussion 28.
5. Fagrell D, Enestrom S, Berggren A, Kniola B. Fat Cylinder Transplantation: An Experimental Comparative Study of Three Different Kinds of Fat Transplants. Plast Reconstr Surg 1996;98:90-6; discussion 7-8.
6. Smahel J. Failure of Adipose Tissue to Heal in the Capsule Preformed by a Silicone Implant. Eur J Plast Surg 1985;8:109-15.
7. Smahel J. Experimental Implantation of Adipose Tissue Fragments. Br J Plast Surg 1989;42:207-11.
8. Tabata Y. The Importance of Drug Delivery Systems in Tissue Engineering. Pharmaceutical Science and Technology Today 2000;3:80-9.
9. Brey EM, Patrick CW, Jr. Tissue Engineering Applied to Reconstructive Surgery. IEEE Eng Med Biol Mag 2000;19:122-5.
10. Patrick CW, Jr. Adipose Tissue Engineering: The Future of Breast and Soft Tissue

Reconstruction Following Tumor Resection. Semin Surg Oncol 2000;19:302-11.

11. Patrick CW, Jr., Mikos AG, McIntire LV. Frontiers in Tissue Engineering. New York: Pergamon, 1998.
12. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies. Tissue Eng 2001;7:211-28.
13. Green H, Kehinde O. Formation of Normally Differentiated Subcutaneous Fat Pads by an Established Preadipose Cell Line. J Cell Physiol 1979;101:169-71.
14. Patrick CW, Jr., Chauvin PB, Hobley J, Reece GP. Preadipocyte Seeded PLGA Scaffolds for Adipose Tissue Engineering. Tissue Eng 1999;5:139-51.
15. von Heimburg D, Zachariah S, Heschel I, Kuhling H, Schoof H, Hafemann B, Pallua N. Human Preadipocytes Seeded on Freeze-Dried Collagen Scaffolds Investigated in Vitro and in Vivo. Biomaterials 2001;22:429-38.
16. von Heimburg D, Zachariah S, Low A, Pallua N. Influence of Different Biodegradable Carriers on the in Vivo Behavior of Human Adipose Precursor Cells. Plast Reconstr Surg 2001;108:411-20; discussion 21-2.
17. Ikada Y, Tabata Y. Protein Release from Gelatin Matrices. Adv Drug Deliv Rev 1998;31:287-301.
18. Tabata Y, Hijikata S, Ikada Y. Enhanced Vascularization and Tissue Granulation by Basic Fibroblast Growth Factor Impregnated in Gelatin Hydrogels. J Control Release 1994;31:189-99.
19. Tabata Y, Hijikata S, Muniruzzaman M, Ikada Y. Neovascularization Effect of Biodegradable Gelatin Microspheres Incorporating Basic Fibroblast Growth Factor.

J Biomater Sci Polym Ed 1999;10:79-94.

20. Tabata Y, Ikada Y. Potentiated in Vivo Biological Activity of Basic Fibroblast Growth Factor by Incorporation Intopolymer Hydrogel Microsphere. 4th Japan International SAMPE Symposium 1995;4:577-82.
21. Tabata Y, Nagano A, Ikada Y. Biodegradation of Hydrogel Carrier Incorporating Fibroblast Growth Factor. Tissue Eng 1999;5:127-38.
22. Tabata Y, Ikada Y. Vascularization Effect of Basic Fibroblast Growth Factor Released from Gelatin Hydrogels with Different Biodegradabilities. Biomaterials 1999;20:2169-75.
23. Caplan AI. Mesenchymal Stem Cells. J Orthop Res 1991;9:641-50.
24. Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF. Promoting Effect of Glucocorticoids on the Differentiation of Human Adipocyte Precursor Cells Cultured in a Chemically Defined Medium. J Clin Invest 1989;84:1663-70.
25. Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E, Debatin KM, Hauner H. Characterization of a Human Preadipocyte Cell Strain with High Capacity for Adipose Differentiation. Int J Obes Relat Metab Disord 2001;25:8-15.
26. Ailhaud G, Grimaldi P, Negrel R. Cellular and Molecular Aspects of Adipose Tissue Development. Annu Rev Nutr 1992;12:207-33.
27. Yuksel E, Weinfeld AB, Cleek R, Waugh JM, Jensen J, Boutros S, Shenaq SM, Spira M. De Novo Adipose Tissue Generation through Long-Term, Local Delivery of Insulin and Insulin-Like Growth Factor-1 by PLGA/PEG Microspheres in an in Vivo Rat Model: A Novel Concept and Capability. Plast Reconstr Surg

2000;105:1721-9.

28. Tabata Y, Miyao M, Inamoto T, Ishii T, Hirano Y, Yamaoki Y, Ikada Y. De Novo Formation of Adipose Tissue by Controlled Release of Basic Fibroblast Growth Factor. Tissue Eng 2000;6:279-89.
29. Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In Vitro Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials 1998;19:1781-9.
30. Broad TE, Ham RG. Growth and Adipose Differentiation of Sheep Preadipocyte Fibroblasts in Serum-Free Medium. Eur J Biochem 1983;135:33-9.

Chapter 2

Effect of material properties on regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor combined with human preadipocytes

INTRODUCTION

In the reconstruction surgery of adipose tissues for the treatments of breast or subcutaneous adipose tissues defect, the grafting of autologous free fat tissue of a few millimeters size and semiliquid has been clinically performed [1, 2]. However, this therapy often meets some problems, such as the absorption and fibrosis of tissues grafted [3-5]. As one trial to tackle the problems, it is important to artificially induce regeneration of adipose tissue at the defect site, which will be a promising therapeutic substitute for the tissue graft.

Recently, tissue engineering has been being noticed as a newly emerging biomedical technology to repair or regenerate body defects by combining stem or precursor cells with their scaffolds and growth factors [6, 7]. To this end, biomaterial design and preparation of scaffolds to promote cell migration and proliferation or growth factor delivery have been extensively tried [6, 8]. It has been demonstrated that the regeneration of adipose tissue by the subcutaneous implantation of collagen

scaffolds and gelatin microspheres containing basic fibroblast growth factor (bFGF) combined with human preadipocytes in Chapter 1. The controlled release technology with the microspheres enabled bFGF to enhance the biological activities of preadipocytes proliferation and neovascularization into the scaffold, resulting in promoted cells-induced regeneration of adipose tissue. The idea is that biomaterials for cell scaffolds, space making membranes, and growth factors delivery give cells a local environment necessary for cell-induced tissue regeneration.

This chapter is undertaken to obtain fundamental information about effect of the biodegradability of collagen sponge scaffolds and the bFGF release profile on the regeneration of adipose tissue. Although the *in vivo* degradability of scaffolds has an important influence on successful tissue regeneration and replacement [9], little has been reported on the topic. In addition, the time profile of bFGF may affect adipose tissue regeneration. In this chapter, collagen sponge scaffolds with different rates of biodegradability were prepared by changing the crosslinking conditions in sponge preparation, while the biodegradation profile of gelatin microspheres was changed to alter the time profile of bFGF release. Following the subcutaneous implantations of collagen sponges and the gelatin microspheres containing bFGF combined with human preadipocytes into the back of nude mice, the histological area of adipose tissue regenerated was evaluated in terms of scaffolds biodegradability and bFGF release profile.

EXPERIMENTAL

Materials

An aqueous solution of human recombinant bFGF with an isoelectric point (IEP) of 9.6 (10 mg/ml) was kindly supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. Collagen solution (pH 3.0, 0.3 wt%) was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. A gelatin sample with an IEP of 5.0 (Nitta Gelatin) was prepared through an alkaline process of type I collagen obtained from the bovine bone. Glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan and used without further purification.

Preparation of collagen scaffolds

Type I porcine collagen solution (10 ml) was poured into an aluminum mold (6 x 6 cm^2) and frozen at -20 °C. Then, the samples were freeze-dried to obtain porous sponges. The freeze-dried sponge was placed in a vacuum oven for dehydrothermal crosslinking of collagen (105 °C, 24 hr, 0.01 Torr), and next immersed to various concentrations of glutaraldehyde solution for various time periods (Table. 1a) for chemical crosslinking at 4 °C. Then, the crosslinked sponges were immersed in 100 mM glycine aqueous solution to block the residual aldehyde groups, and washed with double distilled water (DDW) thoroughly. The resultant sponges were freeze-dried again and sterilized by ethyleneoxide.

Table 1a. Preparation of collagen sponges crosslinked

Code	Dehydrothermal crosslinking	GA concentration [%(w/v)]	Reaction time (hr)
C1	+	-	-
C2	+	0.02	3
C3	+	0.02	24
C4	+	0.2	3
C5	+	0.2	24

Preparation of gelatin microspheres containing bFGF

Gelatin microspheres were prepared through the gelation of gelatin aqueous solution in an emulsion state, followed by the chemical crosslinking with glutaraldehyde solution in the dispersion state. Gelatin aqueous solution (10 ml) preheated at 45 °C was added dropwise to 375 ml of olive oil under stirring at 420 rpm and 45 °C to obtain a W/O emulsion. Stirring was continued at 4 °C for 1 hr to allow gelatin for gelation. After the addition of 100 ml of acetone to the reaction mixture, the resulting microspheres were collected by centrifugation (4 °C, 5,000 rpm, 5 min) and washed 5 times with acetone by centrifugation.

The non-crosslinked and dried gelatin microspheres (500 mg) were placed in 0.1 %(w/v) of Tween 80 aqueous solution containing different concentrations of GA (Table 1b, 100 ml) and stirred at 4 °C for 24 hr to proceed their crosslinking. After collecting by centrifugation (4 °C , 5000 rpm, 5 min), the microspheres were further agitated in 100 ml of 100 mM glycine aqueous solution at 37 °C for 1hr to block the

Table 1b. Preparation of gelatin microspheres crosslinked

Code	GA concentration [%(w/v)]	Reaction time (hr)
G1	0.620	24
G2	1.24	24
G3	6.10	24

residual aldehyde groups of untreated GA. The resulting microspheres were finally washed with DDW, centrifuged at 5000 rpm for 5 min, and freeze-dried.

The original bFGF solution was diluted with DDW to adjust the solution concentration at 50 μg/ml. The aqueous solution of bFGF (20 μl) was dropped onto 2 mg of freeze-dried gelatin microspheres, followed by leaving at 25 °C for 3 hr for impregnation of bFGF into the microspheres. The bFGF solution was completely absorbed into the microspheres through the impregnation process because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres. Tabata *et al.* indicated that the time periods of gelatin microspheres completely degraded *in vivo* were 1, 3, and 5 weeks for G1, G2, and G3 microspheres respectively [10].

Evaluation of biodegradability of collagen scaffolds

In vivo degradation profile of collagen sponge scaffolds was evaluated in terms of radioactivity loss of the ^{125}I-labeled collagen sponges [11]. Briefly, 20 μl of phosphate-buffered saline (PBS, pH 7.4) solution of [^{125}I] Bolton-Hunter reagent (NEX120H, PerkinElmer Inc., Waltham, MA) was homogeneously dropped onto

collagen sponges (5 x 5 x 3 mm^3) for radiolabeling. After incubation of sponges at 4 °C for 3 hr, the sponges were washed with DDW at 4 °C thoroughly, then implanted into the back subcutis of female ddY mice, 6 week-age (Shimizu Laboratory Supply, Kyoto, Japan). Remaining sponges and tissues around the implanted site were obtained at different time intervals and their radioactivity was counted by a gamma counter (ARC-360, Aloka Co., Ltd., Tokyo, Japan) to evaluate the biodegradation profile of collagen sponges.

Isolation and culture of human preadipocytes

Isolation and culture of human preadipocytes were performed by the method described in Chapter 1. The cells were expanded and subjected to the following *in vivo* experiments. The cell morphology was fibroblast-like. When cultured in the presence of 50 nM of insulin, 100 nM of dexamethasone, 10 μg/ml of transferrin, and 200 pM of triiodethyronine for 14 days, the cells accumulated lipid droplets inside. This suggests that the cells isolated had an inherent nature to differentiate into matured adipocytes.

Cell attachment test for collagen scaffolds

The cells compatibility of collagen sponge scaffolds was evaluated by the initial cell attachment of human preadipocytes [12]. Briefly, 500 μl of a cell suspension (2 x 10^6 cells/ml) was poured in a polypropylene tube (2236-012N, Iwaki Glass, Chiba, Japan). A collagen sponge (6 mm diameter, 3 mm thickness) was placed in the cell suspension, followed by agitation with an orbital shaker (Bellco Glass, Vineland, NJ) at 300 rpm for 6 hr. The cell-seeded collagen sponges were thoroughly washed with PBS

to exclude non-adherent cells. The number of cells attached in collagen sponge was determined by the fluorometric quantification assay of cellular DNA [13]. Cell-seeded sponges were digested by the collagenase solution in a water bath at 37 °C for 30 min under shaking. The cells prepared were washed with PBS twice and stored at -20 °C until assayed. After thawing, cells were lysed in 1 ml of a buffer solution (pH 7.4) containing 0.2 mg/ml of sodium dodecylsulfate and 30 mM sodium citrate-buffered saline (SSC) with pipetting. The cell lysate (100 µl) was mixed with 400 µl of SSC buffer. After mixing with 500 µl of a dye solution containing 30 mM SSC and 1 µg/ml of Hoechst 33258 (Nacalai Tesque), the fluorescence intensity of the mixed solution was measured with a fluorescence spectrometer (F-2000, Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 355 and 460 nm, respectively. The percent cells attached was estimated from the calibration curve prepared by use of known initial number of cells.

***In vivo* experiments**

bFGF aqueous solution (20 µl, 0.05 mg/ml) was dropped onto 2 mg of gelatin microspheres freeze-dried, followed by leaving at 25 °C for 3 hr to obtain gelatin microspheres containing bFGF. The microspheres were mixed with the suspension of human preadipocytes at a density of 1 x 10^5 cells/50 µl culture medium. The mixed suspension was dropped on the freeze-dried collagen sponge (10 x 10 x 3 mm^3), followed by incubation at 37 °C for 3 hr and 5 % CO_2 - 95 % air atmosphere pressure to obtain the collagen sponge and gelatin microspheres containing bFGF combined with human preadipocytes.

Under anesthesia, the collagen sponge and gelatin microspheres containing bFGF combined with human preadipocytes was carefully implanted into the back subcutis of female BALB/c nude mice, 6 week-age 1.5 cm apart from the tail root at the body center where is free of originally existing adipose tissue.. Each experimental group was composed of 6 mice. The mice, 4 weeks after implantation, were sacrificed by an overdose injection of anesthetic and the skin including the implanted site (2 x 2 cm^2) was carefully taken off for the subsequent examinations.

Histological analysis

Regeneration of adipose tissues at the site implanted was assessed in terms of histological examination. The skin flap was fixed with 10 % neutralized formalin solution, embedded in paraffin, and sectioned (4 μm in thickness) at the portion of implanted site as central as possible, followed by staining with hematoxylin and eosin (HE). Microphotographs of 6 cross-sections from 6 different mice were taken at a similar magnification to histologically evaluate the *de novo* regeneration of adipose tissue. The same area of interest (3 portions/cross-section, 0.8 x 0.5 mm^2) was randomly selected and the area occupied by matured adipocytes at the implanted site for every portion was measured to express as the area of adipose tissue.

Statistical analysis

All the data were analyzed by Fisher's LSD test for multiple comparison and the statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the mean + standard deviation of the mean (SD).

RESULTS

In vivo degradation profiles of collagen scaffolds

Figure 1 shows the biodegradability of collagen sponges in mice subcutis. The sponges were radiolabeled with the Bolton-Hunter reagent and their biodegradability was evaluated by counting the remaining radioactivity around the implanted site after subcutaneous implantation. In the case of C1 sponges without GA-crosslinking, the sponges disappeared three days after implantation. On the contrary, GA-crosslinked sponges were remained in the body for longer time periods, and the remaining time of radioactivity around the implanted sites changed from 7 to 35 days. The degradation profile of collagen sponges could be controlled by changing GA concentration and crosslinking time. The higher GA concentration and the longer treatment time, the longer time period the sponges were remained.

Cellular compatibility of collagen scaffolds with different biodegradabilities

Figure 2 shows the ratio of human preadipocytes attached into collagen sponges 6 hr after orbital agitation with cell suspension. About 50 % number of cells seeded was attached into any collagen sponge. There was no significant difference in the cell number ratio between all the groups.

Regeneration of adipose tissue by collagen scaffolds and gelatin microspheres with different biodegradabilities

Figure 3 shows the histological sections of C1-C5 collagen sponges combined

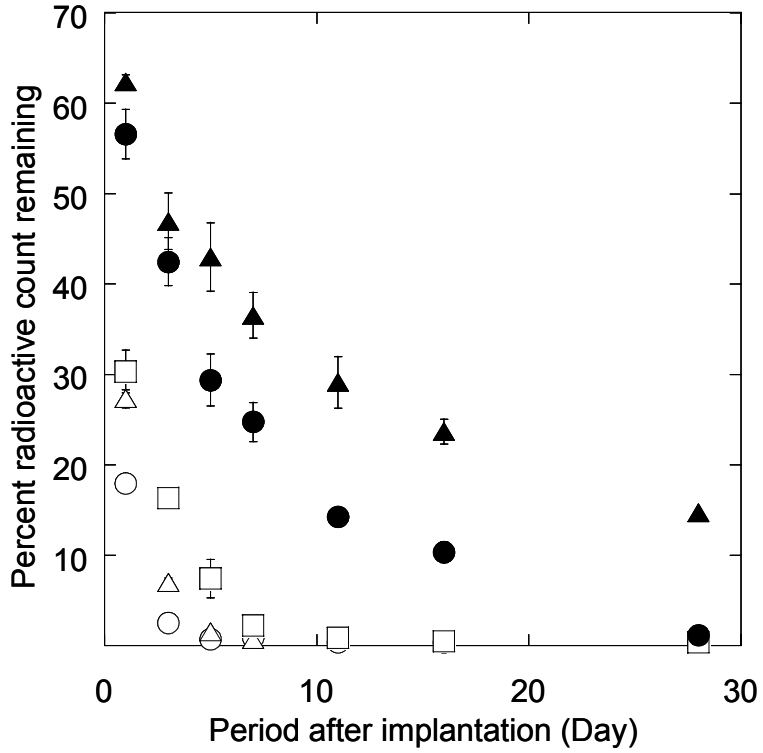

Figure 1. *In vivo* degradation profiles of collagen sponges after implantation into the back subcutis of mice: (○) C1, (△) C2, (□) C3, (●) C4, and (▲) C5 sponges.

with human preadipocytes and G2 gelatin microspheres containing bFGF into back subcutis of nude mice 4 weeks after implantation. Except for the C5 collagen sponge group, matured adipocytes which are indicated as white circular cells in figures and signed as "F", were observed in every group under the muscle layer of mice subcutis, although the number depended on the group. Eosin-stained ribbon-like matrices were observed at the implanted site of C4 and C5 collagen sponge implantation groups.

Figure 4 shows the area of adipose tissues determined from the histological image analysis 4 weeks after implantation of C1-C5 collagen sponge combined with human preadipocytes and G2 gelatin microspheres containing bFGF. The highest area of

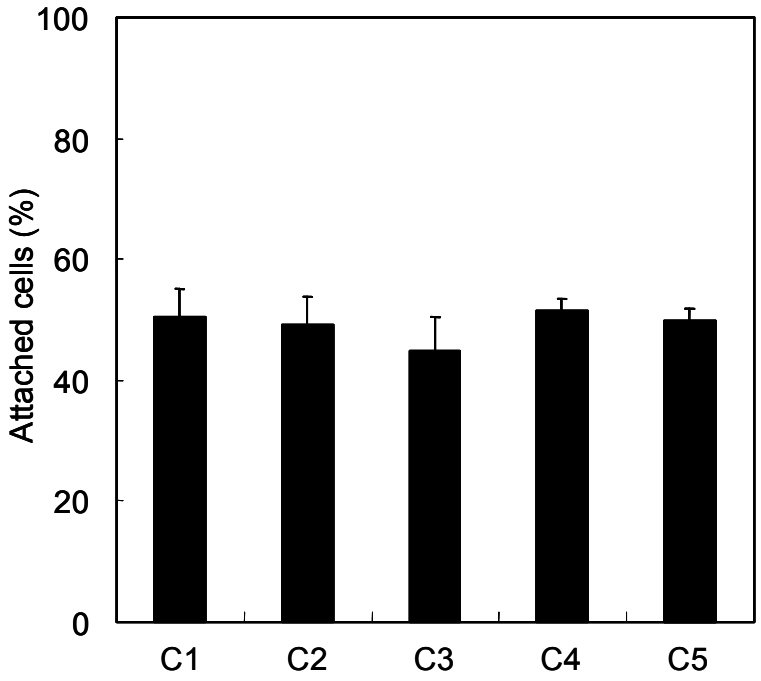

Figure 2. Number of cells attached to collagen sponges 6 hr after agitation cell seeding.

adipose tissues regenerated was observed for the collagen sponge with a biodegradability of two weeks. If the biodegradation period was shorter than two weeks, the cross-sectional area of adipose tissues regenerated was smaller. The area was also smaller for the sponge with longer biodegradation time periods.

Figure 5 shows the histological sections at the implanted site of C3 collagen sponges combined with human preadipocytes and G1-G3 gelatin microspheres containing bFGF. Matured adipocytes were observed in every group under the muscle layer of mice subcutis. Figure 6 shows the area of adipose tissues determined from the histological image analysis 4 weeks after implantation C3 collagen sponges combined with human preadipocytes and G1-G3 gelatin microspheres containing bFGF. There was

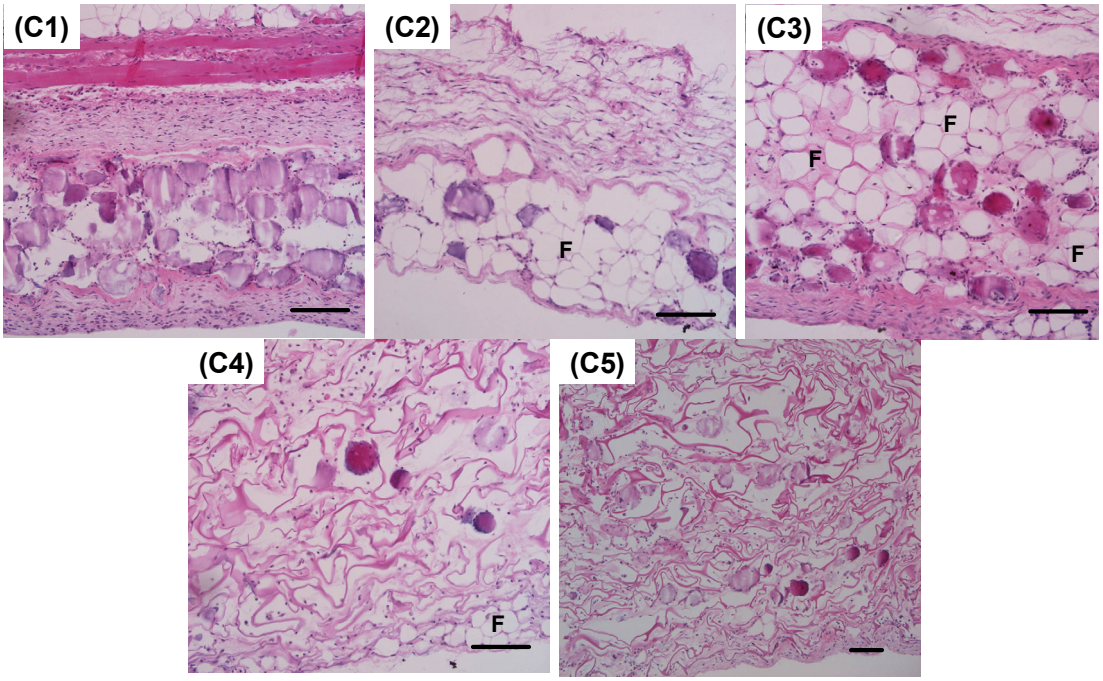

Figure 3. Histological cross-sections of C1, C2, C3, C4, C5 collagen sponges combined with human preadipocytes and G2 gelatin microspheres incorporating bFGF 4 weeks after implantation into the back subcutis of nude mice. F: mature adipocyte. (HE staining, Scale bar = 100 μm)

no significant difference in the cross-sectional area of adipose tissues regenerated among the G1-G3 groups.

DISCUSSION

The effect of sponge scaffold biodegradation and bFGF release profiles on the *de novo* regeneration of adipose tissues was evaluated in this chapter. As the results, an optimal degradation time of scaffolds was found for preadipocyte-induced regeneration

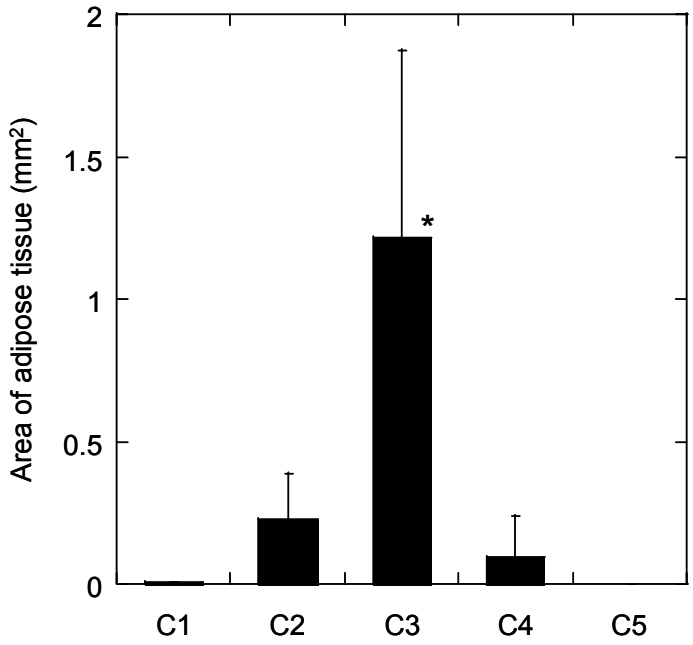

Figure 4. Area of adipose tissues regenerated 4 weeks after implantation of C1, C2, C3, C4, and C5 collagen sponges combined with human preadipocytes and G2 gelatin microspheres incorporating bFGF into the back subcutis of nude mice. *: $p < 0.05$ significant against the area of adipose tissue of other groups.

of adipose tissues. Adipose tissue engineering has been achieved by the implantation of collagen sponge with human preadipocytes and releasing system of bFGF in chapter 1. It is demonstrated that there was an optimal cell number and bFGF dose for *de novo* adipose tissue regeneration.

In the field of tissue engineering, many biomaterials have been used as a matrix for cells scaffolds and bioactive molecules [14, 15]. The controlled release of bioactive molecules also has a key influence on the successful tissue regeneration [8, 16]. For the scaffold, many researches aim at the control of physical property [17, 18], physical stiffness [19, 20], and *in vivo* biodegradation [20-23] to modify the cells behavior. Among them, the scaffold degradation was studied in the area of bone substitute to

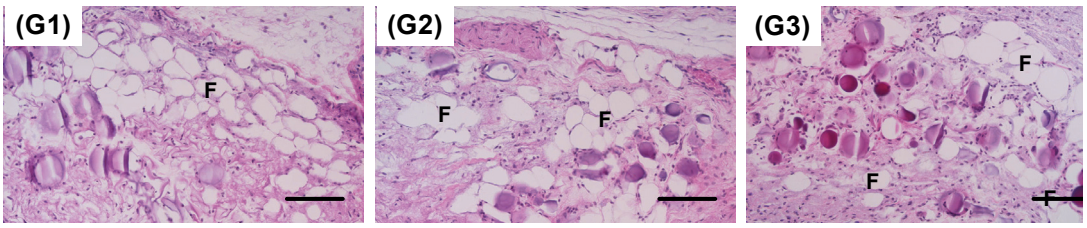

Figure 5. Histological cross-sections of C3 collagen sponges combined with human preadipocytes and G1, G2, and G3 gelatin microspheres incorporating bFGF 4 weeks after implantation into the back subcutis of nude mice. F: mature adipocyte. (HE staining, Scale bar = 100 μm)

achieve the proper contact with native bone tissue and prevent bone absorption around the materials implanted. In the reports, it is concluded that the faster degradation is preferred to bone tissue regeneration and the migration of vascular endothelial cells. However, the effect of scaffold biodegradation on the soft tissue regeneration is not investigated. In this chapter, too fast and too slow degradation of collagen scaffolds resulted in less regeneration of adipose tissues.

In Figure 2, no difference in the cell compatibility among the C1-C5 collagen sponges was observed. Based on this, we could estimate the effect of sponge biodegradability alone on the adipose tissue engineering. For less regeneration of fast degraded sponges, this phenomenon may be explained in terms of the scaffolds property to make a space for tissue regeneration. Generally, it is thought that the scaffold plays two important roles in tissue regeneration; the platform of cells attachment and maintenance, and the space making for tissue regeneration. The latter has been clinically confirmed through the guided tissue regeneration (GTR) application to dental surgery. The GTR membrane made from non-biodegradable material was used for space-making

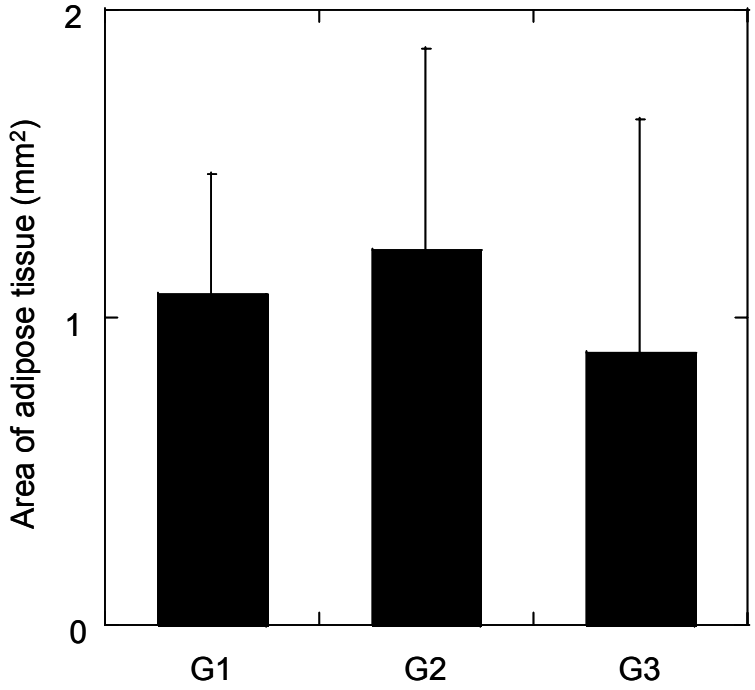

Figure 6. Area of adipose tissues regenerated 4 weeks after implantation of C3 collagen sponges combined with human preadipocytes and G1, G2, and G3 gelatin microspheres incorporating bFGF into the back subcutis of nude mice.

and was effective for prevention of scar tissue ingrowth [24]. Taken together, it is possible that too fast degradation of scaffold could not provide the space for growth and differentiation of preadipocytes. Slow degradation of collagen sponges contributed to less regeneration of adipose tissue. The phenomenon may be explained in terms of physical impairment of tissue regeneration process. The collagen sponge implanted is basically not biological natural, but foreign and artificial material, although, it is reported to function as a scaffold suitable for the initial step of regeneration. It is conceivable that the remaining in the site to be regenerated physically impairs the natural process of tissue regeneration, resulting in less regeneration of adipose tissues. The balance of scaffold degradation is key to induce cell-based tissue regeneration.

There was no significant effect of bFGF release profiles of 1, 3, and 5 weeks on the adipose tissue regeneration. It has been demonstrated that gelatin microspheres containing bFGF were effective in inducing regeneration of adipose tissue in Chapter 1. Implantation of collagen sponges combined with human preadipocytes and gelatin microspheres for the controlled release of bFGF achieved significantly high amount of adipose tissue regenerated compared with the solution injection of bFGF at 10 times higher the dose. Upon implanting with the collagen sponge incorporating either gelatin microspheres containing bFGF or human preadipocytes and the mixture of human preadipocytes and gelatin microspheres containing bFGF, such a change of tissue appearance was not observed at the implanted site. This result indicated that every combination of two from three components, collagen sponges, human preadipocytes, and gelatin microspheres containing bFGF, resulted in no regeneration of adipose tissue. Therefore, three combination of collagen sponge, human preadipocytes, and bFGF was indispensable for adipose tissue regeneration, and the local environment which allows human preadipocytes to proliferate and differentiate into matured adipocytes was provided by implantation of the collagen sponge together with the release system of bFGF. bFGF is known as a promoting factor of preadipocytes growth [25, 26], but controversial about the ability to induce adipogenic differentiation of preadipocytes and other cells [27-29]. On the other hand, bFGF is a strong angiogenic factor *in vivo* [30, 31]. It is possible that bFGF released acts on the initial step of *de novo* regeneration of adipose tissue. The bFGF would induce angiogenesis and the proliferation of preadipocytes implanted. As the result, no significant difference in the regeneration of adipose tissue would be observed by prolonged bFGF release.

This chapter clearly indicates importance of material design to achieve the optimal regeneration of adipose tissues. As expected, the *in vivo* degradability of scaffolds greatly affected the regeneration, because the existence of cell local environment undoubtedly modifies the cell behavior. In addition to the scaffold degradability, the chemical composition and the combination of signaling molecules must be important. This is presently under investigation.

REFERENCES

1. Billings E, Jr., May JW, Jr. Historical Review and Present Status of Free Fat Graft Autotransplantation in Plastic and Reconstructive Surgery. Plast Reconstr Surg 1989;83:368-81.
2. Ellenbogen R. Free Autogenous Pearl Fat Grafts in the Face--a Preliminary Report of a Rediscovered Technique. Ann Plast Surg 1986;16:179-94.
3. Ersek RA. Transplantation of Purified Autologous Fat: A 3-Year Follow-up Is Disappointing. Plast Reconstr Surg 1991;87:219-27; discussion 28.
4. Fagrell D, Enestrom S, Berggren A, Kniola B. Fat Cylinder Transplantation: An Experimental Comparative Study of Three Different Kinds of Fat Transplants. Plast Reconstr Surg 1996;98:90-6; discussion 7-8.
5. Smahel J. Experimental Implantation of Adipose Tissue Fragments. Br J Plast Surg 1989;42:207-11.
6. Langer R. Tissue Engineering: Perspectives, Challenges, and Future Directions. Tissue Eng 2007;13:1-2.
7. Tabata Y. Significance of Release Technology in Tissue Engineering. Drug Discov Today 2005;10:1639-46.
8. Lutolf MP, Hubbell JA. Synthetic Biomaterials as Instructive Extracellular Microenvironments for Morphogenesis in Tissue Engineering. Nat Biotechnol 2005;23:47-55.
9. Langer R, Vacanti JP. Tissue Engineering. Science 1993;260:920-6.
10. Tabata Y, Ikada Y. Vascularization Effect of Basic Fibroblast Growth Factor

Released from Gelatin Hydrogels with Different Biodegradabilities. Biomaterials 1999;20:2169-75.

11. Ozeki M, Tabata Y. In Vivo Degradability of Hydrogels Prepared from Different Gelatins by Various Cross-Linking Methods. J Biomater Sci Polym Ed 2005;16:549-61.
12. Yasuda K, Inoue S, Tabata Y. Influence of Culture Method on the Proliferation and Osteogenic Differentiation of Human Adipo-Stromal Cells in Nonwoven Fabrics. Tissue Eng 2004;10:1587-96.
13. Rao J, Otto WR. Fluorimetric DNA Assay for Cell Growth Estimation. Anal Biochem 1992;207 186-.
14. Tessmar JK, Gopferich AM. Matrices and Scaffolds for Protein Delivery in Tissue Engineering. Adv Drug Deliv Rev 2007;59:274-91.
15. Yang S, Leong KF, Du Z, Chua CK. The Design of Scaffolds for Use in Tissue Engineering. Part I. Traditional Factors. Tissue Eng 2001;7:679-89.
16. Matsumoto T, Mooney DJ. Cell Instructive Polymers. Adv Biochem Eng Biotechnol 2006;102:113-37.
17. O'Brien FJ, Harley BA, Yannas IV, Gibson L. Influence of Freezing Rate on Pore Structure in Freeze-Dried Collagen-GAG Scaffolds. Biomaterials 2004;25:1077-86.
18. Karageorgiou V, Kaplan D. Porosity of 3D Biomaterial Scaffolds and Osteogenesis. Biomaterials 2005;26:5474-91.
19. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell 2006;126:677-89.
20. Urech L, Bittermann AG, Hubbell JA, Hall H. Mechanical Properties, Proteolytic

Degradability and Biological Modifications Affect Angiogenic Process Extension into Native and Modified Fibrin Matrices in Vitro. Biomaterials 2005;26:1369-79.

21. Alsberg E, Kong HJ, Hirano Y, Smith MK, Albeiruti A, Mooney DJ. Regulating Bone Formation via Controlled Scaffold Degradation. J Dent Res 2003;82:903-8.
22. Byrne DP, Lacroix D, Planell JA, Kelly DJ, Prendergast PJ. Simulation of Tissue Differentiation in a Scaffold as a Function of Porosity, Young's Modulus and Dissolution Rate: Application of Mechanobiological Models in Tissue Engineering. Biomaterials 2007;28:5544-54.
23. Milella E, Brescia E, Massaro C, Ramires PA, Miglietta MR, Fiori V, Aversa P. Physico-Chemical Properties and Degradability of Non-Woven Hyaluronan Benzylic Esters as Tissue Engineering Scaffolds. Biomaterials 2002;23:1053-63.
24. Wang HL, Greenwell H, Fiorellini J, Giannobile W, Offenbacher S, Salkin L, Townsend C, Sheridan P, Genco RJ. Periodontal Regeneration. J Periodontol 2005;76:1601-22.
25. Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De Novo Adipogenesis in Mice at the Site of Injection of Basement Membrane and Basic Fibroblast Growth Factor. Proc Natl Acad Sci U S A 1998;95:1062-6.
26. Broad TE, Ham RG. Growth and Adipose Differentiation of Sheep Preadipocyte Fibroblasts in Serum-Free Medium. Eur J Biochem 1983;135:33-9.
27. Locklin RM, Oreffo RO, Triffitt JT. Effects of TGFbeta and bFGF on the Differentiation of Human Bone Marrow Stromal Fibroblasts. Cell Biol Int 1999;23:185-94.
28. Neubauer M, Fischbach C, Bauer-Kreisel P, Lieb E, Hacker M, Tessmar J, Schulz

MB, Goepferich A, Blunk T. Basic Fibroblast Growth Factor Enhances PPARgamma Ligand-Induced Adipogenesis of Mesenchymal Stem Cells. FEBS Lett 2004;577:277-83.

29. Roncari DA, Le Blanc PE. Inhibition of Rat Perirenal Preadipocyte Differentiation. Biochem Cell Biol 1990;68:238-42.
30. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR. A Simple, Quantitative Method for Assessing Angiogenesis and Antiangiogenic Agents Using Reconstituted Basement Membrane, Heparin, and Fibroblast Growth Factor. Lab Invest 1992;67:519-28.
31. Tabata Y, Hijikata S, Ikada Y. Enhanced Vascularization and Tissue Granulation by Basic Fibroblast Growth Factor Impregnated in Gelatin Hydrogels. J Control Release 1994;31:189-99.

Chapter 3

Effect of chemical composition of scaffold materials on proliferation and adipogenic differentiation of human preadipocytes

INTRODUCTION

Recently, the regeneration of adipose tissues has been induced by using the technology and methodology of tissue engineering [1]. This technique needs precursor cells, as well as the scaffolds for cell attachment, the space making, and bio-signaling molecules to survive the cells and maintain their functions. As the scaffold materials, poly (glycolide-co-lactide) [2], poly(glycolic acid) [3], poly(ethyleneglycol diacrylate) [4], type I collagen [5], hyaluronic acid [6], fibrin [7], fibroin [8], Matrigel® [9], and decellularized matrices [10] have been used. These scaffolds should have various properties, such as the moldability, physical property, biocompatibility, and bioabsorbability. Little has been reported on cell scaffolds with all requirements at present.

The regeneration of adipose tissue by using human preadipocytes, the sponge of type I collagen as a scaffold, and the controlled release of basic fibroblast growth factor (bFGF) from gelatin hydrogel microspheres for preadipocytes growth and neovascularization has been developed. A sponge of type I collagen used has been

clinically used as an artificial dermis for about twenty years to prove the biocompatibility and biosafety [11]. The type I collagen is ubiquitously present in our body and one of the main components of extracellular matrix (ECM). Moreover, it has a cell-binding domain for the biological maintenance of various cells [12, 13]. On the contrary, it was reported that there are many ECM components in native adipose tissues, such as various types of collagen, laminin, and others [14]. In addition, several studies reported that the change of protein expression and secretion from preadipocytes was detected with their adipogenic differentiation [15-18]. Based on these findings, it is possible that the incorporation of the ECM components into type I collagen affects the behavior and biological functions of preadipocytes, resulting in cell-based regeneration of adipose tissue. This chapter was undertaken to investigate the effect of ECM components incorporated to type I collagen on the proliferation and adipogenic differentiation of human preadipocytes. The components and type I collagen were coated on polystyrene cell culture plates at different mixing ratios. The proliferation and differentiation of human preadipocyte on the coated plates were evaluated.

EXPERIMENTAL

Materials

Type I porcine tendon and type IV bovine lens collagen solutions (pH 3.0, 0.3 wt%) were kindly supplied by Nitta Gelatin Inc., Osaka, Japan. Laminin-1 (354232, lot No. 30747) was purchased from BD Bioscience (San Jose, CA). Hyaluronic acid

sodium salt (Mw: 1,980,000) was kindly supplied by Denkikagaku Kogyo Co. Ltd. (Tokyo, Japan). A gelatin sample with an isoelectric point of 5.0 (Nitta Gelatin) was prepared through an alkaline process of type I collagen obtained from the bovine bone. $Na^{125}I$ (NEZ-033H, >12.95 GBq/ml) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan and used without further purification.

Coating of various ECM components with type I collagen

Type I collagen solution (10 μg/ml) was mixed at 4 °C with 10 μg/ml of type IV collagen, laminin-1, hyaluronic acid, and gelatin at volume ratios of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:10. The mixed solutions were cast on each well of polystyrene multiwell cell culture plate (50 μl for 96 well and 200 μl for 24 well ,Corning Inc., Lowell, MA) and incubated for 12 hr at room temperature. The plates coated were washed three times with 10 mM of phosphate-buffered saline solution (PBS, pH 7.4) and used for further experiment.

Evaluation of amounts of mixed ECM components and type I collagen coated

Type IV collagen, laminin-1, hyaluronic acid, and gelatin were radioiodinated according to the conventional chloramine T method as previously described [19]. Briefly, 5 μl of $Na^{125}I$ was added to 200 μl of protein solution in 0.5 M potassium phosphate-buffer (pH 7.5) containing 0.5 M sodium chloride. Then, 0.2 mg/ml of chloramine-T in same buffer (100 μl) was added to the solution mixture. After agitation at room temperature for 2 min, 100 μl of PBS containing 0.4 mg of sodium

metabisulfate was added to the reaction solution to stop the radioiodination. The reaction mixture was passed through a PD-10 column (GE Healthcare Life Sciences, Giles, U.K.) to remove the uncoupled, free ^{125}I molecules from the ^{125}I-labeled protein. The concentration of radioiodinated type I collagen was determined by using Sircol collagen assay kit (Biocolor Ltd, Carrickfergus, U.K.). The concentration of other radioiodinated proteins was determined by using micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Final concentration of the proteins was adjusted at 10 μg/ml by adding the fresh protein stock solution.

The coating of components mixed with type I collagen on the 96-multiwell cell culture plate was performed as described above, and 24 hr later the radioactivity of each well was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan) to evaluate the amount of each component coated. To detect hyaluronic acid, the supernatant after coating was sampled and the concentration was measured by hyaluronan assay kit (Seikagaku Co., Tokyo, Japan) according to the manufacturer's instruction.

Isolation of human preadipocytes

Isolation and culture of human preadipocytes were performed by the method described in Chapter 1. The cell morphology was fibroblast-like. When cultured in the presence of 50 nM of insulin, 100 nM of dexamethasone, 10 μg/ml of transferrin, and 200 pM of triiodethyronine for 14 days, the cells accumulated lipid droplets inside. This suggests that the cells isolated had an inherent nature to differentiate into mature adipocytes. The cells were subcultured to expand for the following experiments.

Evaluation of cell proliferation on the culture plates coated with ECM components and type I collagen

Human preadipocytes were plated into each well of 96-multiwell cell culture plates coated with various ECM components and type I collagen at a density of 1 x 10^4 cells/cm^2. The growth medium was changed every two days. The cell number was evaluated 7 days after cell plating. After addition of Cell Count Reagent SF (10 μl, Nacalai Tesque, Kyoto, Japan) to each well, cells were incubated for 90 min at 37 °C and 5 % CO_2 - 95 % air atmosphere pressure. Then, the absorbance at 450 nm was measured. The absorbance was normalized by that of cells proliferated on non-coated, original cell culture plates to express as proliferation ratio.

Evaluation of adipogenic differentiation on the culture plates coated with ECM components and type I collagen

Human preadipocytes were plated into each well of 24-multiwell cell culture plates coated with various ECM components and type I collagen at a density of 3 x 10^4 cells/cm^2. For adipogenic differentiation, 24 hr after cell plating, the growth medium was replaced to adipogenic differentiation medium composed of DME/Ham's F12 medium (250 μl/cm^2) containing 0.05 μM insulin, 0.2 nM 3,5,3'-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone [20]. After culturing in the differentiation medium further for 14 days, cells were washed three times with PBS and the glycerol-3-phosphate dehydrogenase (GPDH) activity of cells was measured by GPDH assay kit (Primary Cell Co., Ltd., Ishikari, Japan) Briefly, cells were homogenized in a buffer solution of kit using a handy sonic

(UR-20, Tomy Seiko Co., Ltd., Tokyo, Japan) on ice. After mixing a reaction solution of kit, the absorbance of solution mixture was measured at 340 nm by the spectrophotometer (Versa max, Molecular Devices Inc., Union City, CA) to assess the GPDH activity.

Statistical analysis

All the experiments were triplicated, and all the results were statistically analyzed by the unpaired Student's t test. $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean $\pm$ the standard deviation of the mean (SD).

RESULTS

The amount of mixed ECM components and type I collagen coated

Figure 1 shows the amount of ECM components mixed with type I collagen coated on polystyrene cell culture plates. By changing the mixing ratio of ECM components to type I collagen in coating, the amount of proteins coated on the culture plates was changed. Neither precipitation nor change of solution turbidity was observed during the mixing and coating procedures. The amount of hyaluronic acid coated was depended on that of type I collagen.

Proliferation of human preadipocytes on the culture plates coated with ECM components and type I collagen

Figure 2 shows the relative proliferation ratio of human preadipocytes 7 days after culturing on the culture plates coated with ECM components and type I collagen.

The proliferation rate of human preadipocytes was about 1.4-fold higher on the plate coated with type I collagen than on the non-coated, original cell culture plates. Mixed coating of type IV collagen and type I collagen enhanced the preadipocytes proliferation to a significantly great extent compared with that of type I collagen alone. The proliferation was significantly inhibited by coating of hyaluronic acid, laminin-1 or the mixture of gelatin and type I collagen.

Adipogenic differentiation of human preadipocytes on the culture plates coated with ECM components and type I collagen

Figure 3 shows the GPDH activity of human preadipocytes 14 days after culturing in the differentiation medium on the culture plates coated with ECM components and type I collagen. Significantly higher activity of GPDH was observed for human preadipocytes cultured on the culture plates coated with type IV collagen or laminin-1 combined with type I collagen. With an increase in the mixing ratio of type IV collagen to type I collagen, the GPDH activity was increased. On the contrary, the enhanced GPDH activity was observed for mixed laminin-1 and type I collagen. No significant change in the GPDH activity was observed for the culture plates coated with type I collagen plus gelatin or hyaluronic acid.

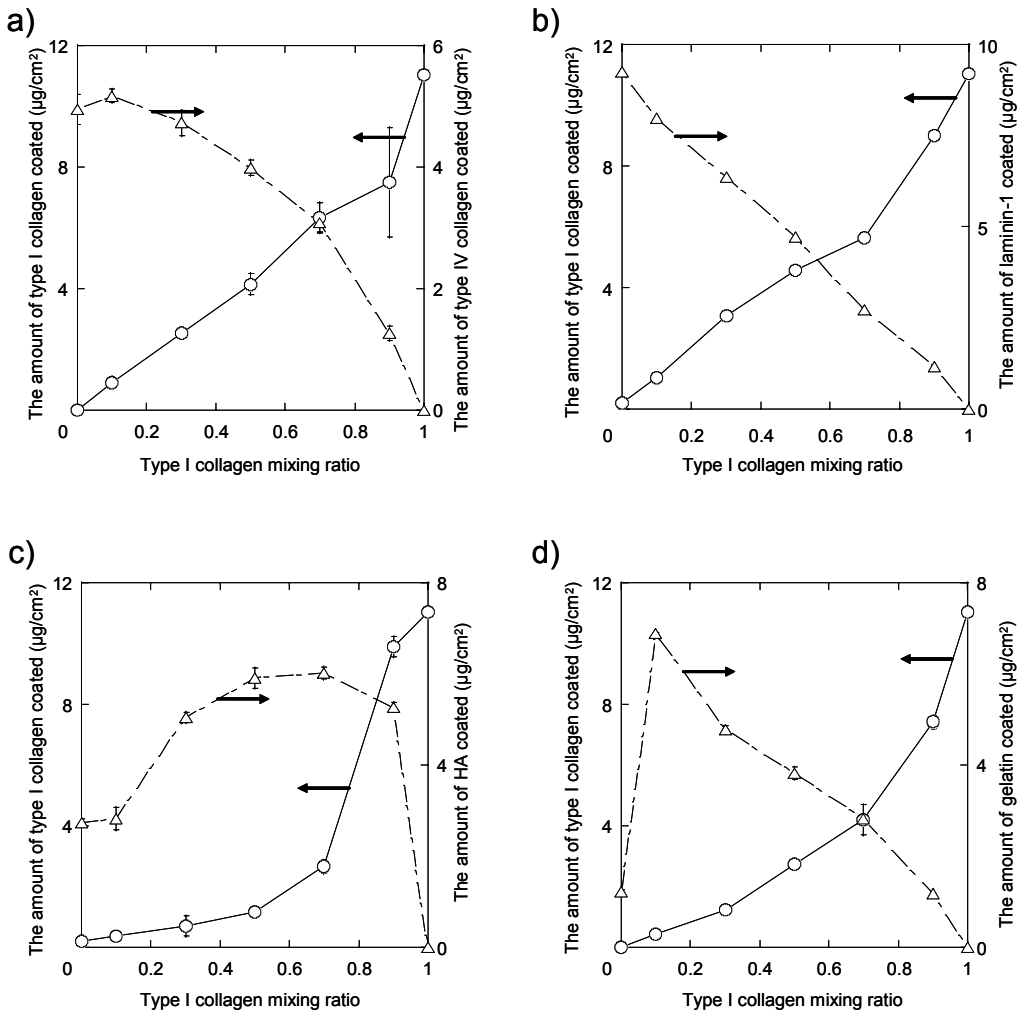

Figure 1. The amount of type IV collagen (a), laminin-1 (b), hyaluronic acid (HA) (c), and gelatin (d) mixed with type I collagen coated on polystyrene cell culture plates as a function of the type I collagen mixing ratio in coating.

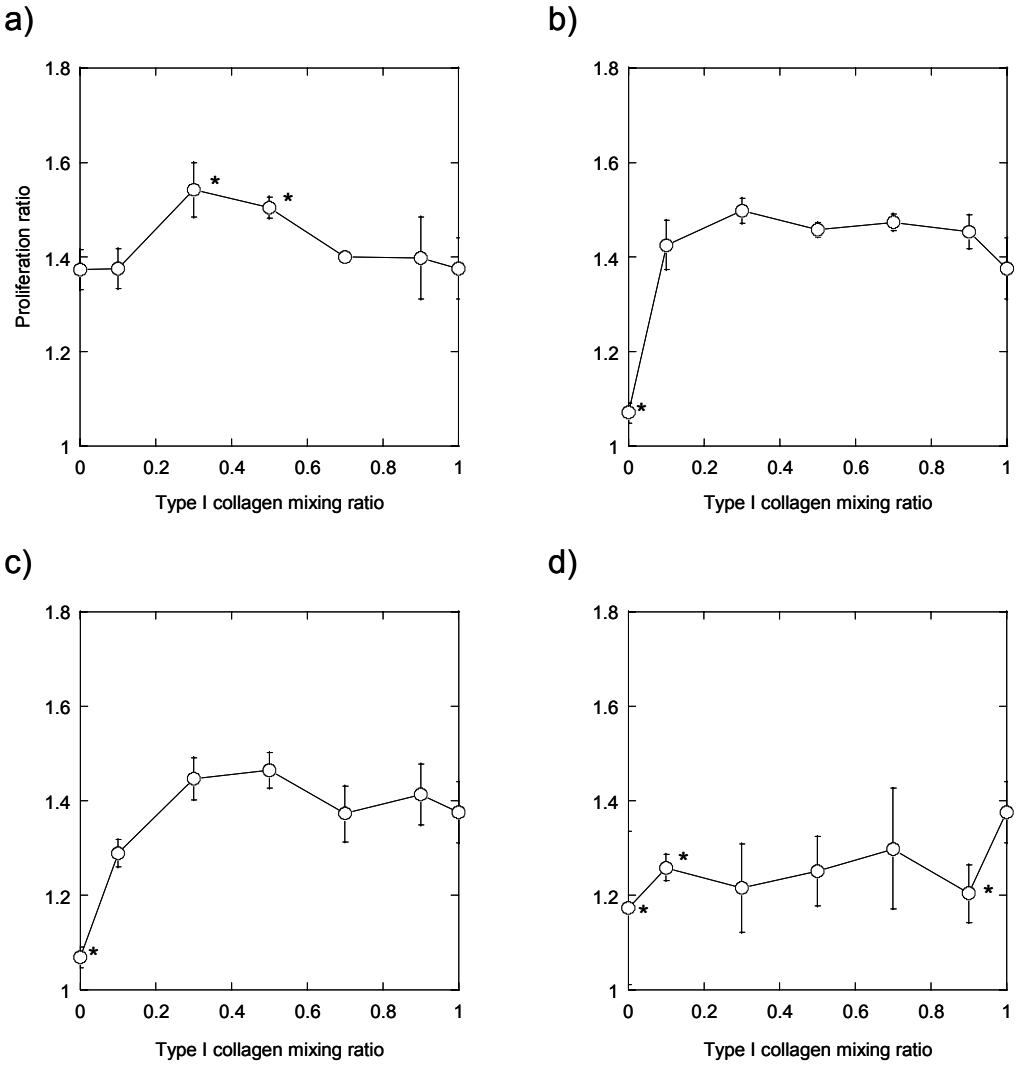

Figure 2. Relative proliferation ratio of human preadipocytes 7 days after culturing on polystyrene cell culture plates coated with type IV collagen (a), laminin-1 (b), hyaluronic acid (HA) (c), gelatin (d) and type I collagen at different mixing ratios. *: $p<0.05$, significant against the proliferation ratio cultured on the culture plates coated with type I collagen.

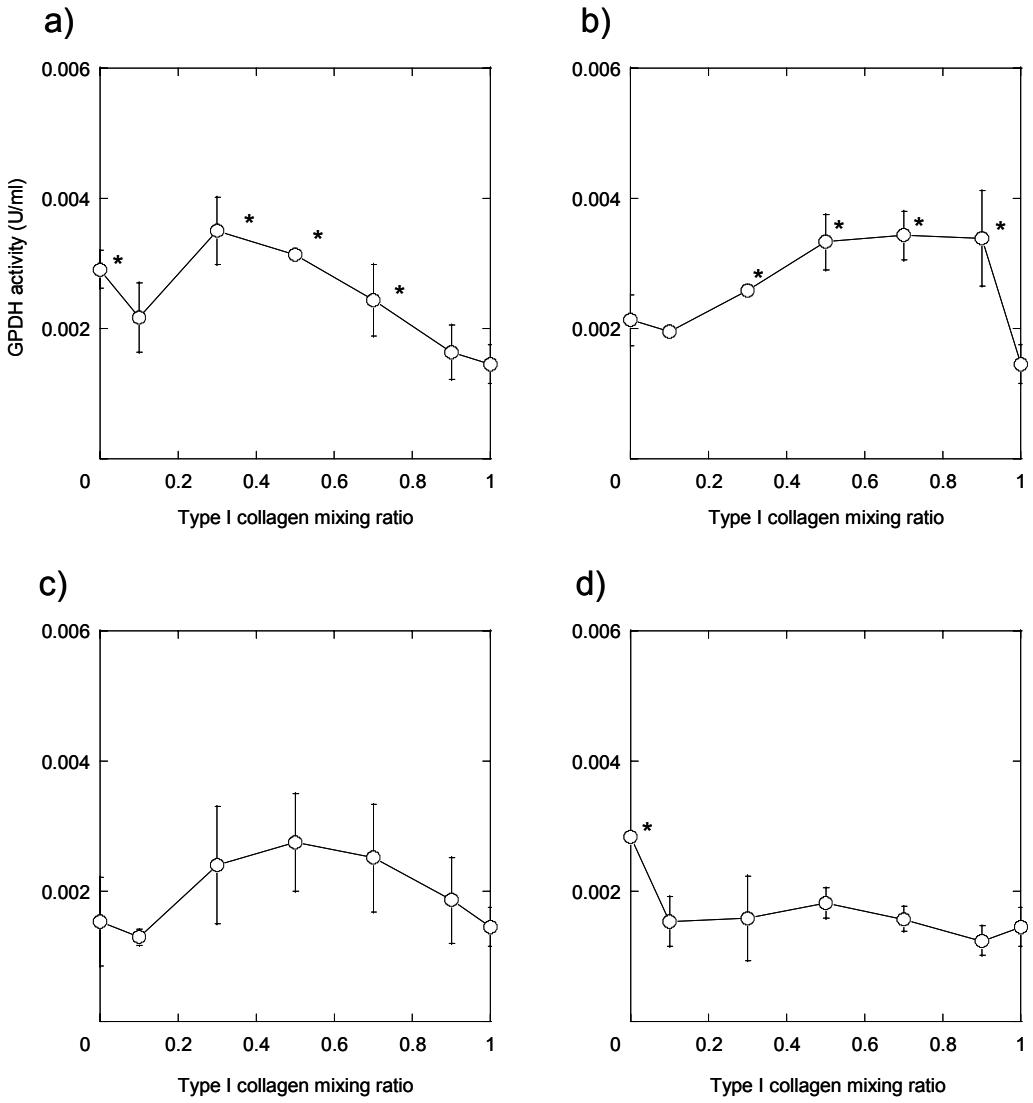

Figure 3. Glycerol-3-phosphate dehydrogenase (GPDH) activity of human preadipocytes 14 days cultured with adipogenic differentiation medium on on polystyrene cell culture plates coated with type IV collagen (a), laminin-1 (b), hyaluronic acid (HA) (c), gelatin (d) and type I collagen at different mixing ratios. *: $p<0.05$, significant against the activity cultured on the culture plates coated with type I collagen.

DISCUSSION

This chapter demonstrates that the ECM components mixing with type I collagen affects the cell behavior of human preadipocytes. The amount of ECM components coated was varied by the mixing ratio to type I collagen, except for hyaluronic acid (Figure 1c). It is reported that type I collagen interacts with hyaluronic acid in a biologically specific manner [21]. It is possible that this specific interaction causes the non-linear relationship in the amount of hyaluronic acid between the added and coated. The amount of gelatin coated was less than that of gelatin mixed with type I collagen (Figure 1d). This phenomenon may be caused by an interaction between the gelatin and type I collagen.

Coating of type IV and type I collagen mixtures significantly enhanced both the proliferation and adipogenic differentiation of human preadipocytes (Figures 2a and 3a). The coating of mixed laminin-1 and type I collagen significantly promoted the adipogenic differentiation (Figure 3b). It is well known that type IV collagen and laminin-1 are the main components of basement membrane [22]. Napolitano *et al.* demonstrates that loculated adipocytes are histologically surrounded by the basement membrane [14]. Kubo *et al.* reported that the surrounding tissue of adipocytes was reactive for anti-type IV collagen and laminin antibodies [17]. In addition, the coating with Matrigel® of basement membrane extract enhanced the adipogenic differentiation of preadipocytes [23]. These findings indicate that the components of basement membrane are necessary for the existence of mature adipocytes. Taken together, it is conceivable that the existence of the components in the process of adipogenic

differentiation affects the differentiation behavior of preadipocytes. However, little has been reported on the effect of type IV collagen and laminin-1 and their combination with type I collagen on the proliferation and adipogenic differentiation. From the viewpoint of material design to regulate the proliferation and differentiation of preadipocytes, the composition and mixing ratio of ECM components on the preadipocyte behavior should be investigated systemically.

In addition to type IV collagen and laminin, hyaluronic acid and gelatin were evaluated for the influence on the behavior of human preadipocytes. Because they have been used as the material of cell scaffold for tissue engineering. The hydrogel and sponge forms of hyaluronic acid have been reported to be effective for the functional maintenance of stem cells [24] and *in vivo* regeneration of adipose tissue [6]. Gelatin is a denaturated type I collagen and applicable to a carrier of bFGF which stimulates proliferation of human preadipocytes [25] and angiogenesis [26]. However, no acceleration effect in the proliferation and adipogenic differentiation of human preadipocytes was observed for hyaluronic acid and gelatin. In this chapter, hyaluronic acid was used as a two-dimensional substrate for human preadipocytes. This dimensional difference may cause different behavior of preadipocytes. Meanwhile, gelatin does not have any higher order structures, like the helix of collagen molecules. It is possible that this structural change affects the biological stimulation to preadipocytes, resulting in no active functions to the cells.

Single coating of hyaluronic acid and laminin-1 had less activity for proliferation of human preadipocytes (Figures 2b and 2c), which is different from that of type IV collagen, gelatin, and type I collagen. It is reported that laminin-1 has an

activity for spreading of preadipocytes [27]. On the contrary, there has been no report published about the activity of hyaluronic acid for proliferation of preadipocytes. The reason is not clear at present. There have been reported on several factors affecting the proliferation and differentiation of preadipocytes. The proliferation of preadipocytes was enhanced by the exposure to bFGF [25], galectin-3 [28], low density lipoprotein [29], and 17beta-estradiol [30] or the co-culturing with endothelial cells [31]. It has been demonstrated that adipogenic differentiation was affected by the bio-signaling molecules of growth factors [32, 33], mechanical stimuli, such as cell spreading area [34], hypoxic conditions [35], and the surface chemistry of substrates [16, 23, 36]. However, at present, it is not clear which factors or their combination is effective in modifying the proliferation and differentiation of cells. The dimension of materials, such as plates and sponges, also affects the cell behavior. Based on the results obtained with the two-dimensional system in this chapter, the sponge scaffold of three-dimension will be designed and prepared to evaluate the effect of ECM components and the combination on the preadipocytes behaviors. Further experiments are now undertaken to create the cell scaffolds for the *in vivo* regeneration of adipose tissue.

REFERENCES

1. Gomillion CT, Burg KJ. Stem Cells and Adipose Tissue Engineering. Biomaterials 2006;27:6052-63.
2. Patrick CW, Jr., Chauvin PB, Hobley J, Reece GP. Preadipocyte Seeded PLGA Scaffolds for Adipose Tissue Engineering. Tissue Eng 1999;5:139-51.
3. Lin SD, Wang KH, Kao AP. Engineered Adipose Tissue of Predefined Shape and Dimensions from Human Adipose-Derived Mesenchymal Stem Cells. Tissue Eng Part A 2008;14:571-81.
4. Alhadlaq A, Tang M, Mao JJ. Engineered Adipose Tissue from Human Mesenchymal Stem Cells Maintains Predefined Shape and Dimension: Implications in Soft Tissue Augmentation and Reconstruction. Tissue Eng 2005;11:556-66.
5. von Heimburg D, Zachariah S, Heschel I, Kuhling H, Schoof H, Hafemann B, Pallua N. Human Preadipocytes Seeded on Freeze-Dried Collagen Scaffolds Investigated in Vitro and in Vivo. Biomaterials 2001;22:429-38.
6. Hemmrich K, Van de Sijpe K, Rhodes NP, Hunt JA, Di Bartolo C, Pallua N, Blondeel P, von Heimburg D. Autologous in Vivo Adipose Tissue Engineering in Hyaluronan-Based Gels--a Pilot Study. J Surg Res 2008;144:82-8.
7. Schoeller T, Lille S, Wechselberger G, Otto A, Mowlavi A, Piza-Katzer H. Histomorphologic and Volumetric Analysis of Implanted Autologous Preadipocyte Cultures Suspended in Fibrin Glue: A Potential New Source for Tissue Augmentation. Aesthetic Plast Surg 2001;25:57-63.
8. Mauney JR, Nguyen T, Gillen K, Kirker-Head C, Gimble JM, Kaplan DL.

Engineering Adipose-Like Tissue in Vitro and in Vivo Utilizing Human Bone Marrow and Adipose-Derived Mesenchymal Stem Cells with Silk Fibroin 3D Scaffolds. Biomaterials 2007;28:5280-90.

9. Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De Novo Adipogenesis in Mice at the Site of Injection of Basement Membrane and Basic Fibroblast Growth Factor. Proc Natl Acad Sci U S A 1998;95:1062-6.
10. Flynn L, Prestwich GD, Semple JL, Woodhouse KA. Adipose Tissue Engineering with Naturally Derived Scaffolds and Adipose-Derived Stem Cells. Biomaterials 2007;28:3834-42.
11. Yannas IV, Burke JF. Design of an Artificial Skin. I. Basic Design Principles. J Biomed Mater Res 1980;14:65-81.
12. Glowacki J, Mizuno S. Collagen Scaffolds for Tissue Engineering. Biopolymers 2008;89:338-44.
13. Cen L, Liu W, Cui L, Zhang W, Cao Y. Collagen Tissue Engineering: Development of Novel Biomaterials and Applications. Pediatr Res 2008;63:492-6.
14. Napolitano LM. Observations on the Fine Structure of Adipose Cells. Ann N Y Acad Sci 1965;131:34-42.
15. Nakajima I, Yamaguchi T, Ozutsumi K, Aso H. Adipose Tissue Extracellular Matrix: Newly Organized by Adipocytes During Differentiation. Differentiation 1998;63:193-200.
16. Tsuruga H, Kumagai H, Kojima T, Kitamura T. Identification of Novel Membrane and Secreted Proteins Upregulated During Adipocyte Differentiation. Biochem Biophys Res Commun 2000;272:293-7.

17. Kubo Y, Kaidzu S, Nakajima I, Takenouchi K, Nakamura F. Organization of Extracellular Matrix Components During Differentiation of Adipocytes in Long-Term Culture. In Vitro Cell Dev Biol Anim 2000;36:38-44.
18. Aratani Y, Kitagawa Y. Enhanced Synthesis and Secretion of Type IV Collagen and Entactin During Adipose Conversion of 3T3-L1 Cells and Production of Unorthodox Laminin Complex. J Biol Chem 1988;263:16163-9.
19. Ozeki M, Tabata Y. In Vivo Degradability of Hydrogels Prepared from Different Gelatins by Various Cross-Linking Methods. J Biomater Sci Polym Ed 2005;16:549-61.
20. Hauner H, Rohrig K, Petruschke T. Effects of Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF) and Fibroblast Growth Factor (FGF) on Human Adipocyte Development and Function. Eur J Clin Invest 1995;25:90-6.
21. Junqueira LC, Montes GS. Biology of Collagen-Proteoglycan Interaction. Arch Histol Jpn 1983;46:589-629.
22. LeBleu VS, Macdonald B, Kalluri R. Structure and Function of Basement Membranes. Exp Biol Med (Maywood) 2007;232:1121-9.
23. O'Connor KC, Song H, Rosenzweig N, Jansen DA. Extracellular Matrix Substrata Alter Adipocyte Yield and Lipogenesis in Primary Cultures of Stromal-Vascular Cells from Human Adipose. Biotechnology letters 2003;25:1967-72.
24. Gerecht S, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic Acid Hydrogel for Controlled Self-Renewal and Differentiation of Human Embryonic Stem Cells. Proc Natl Acad Sci U S A 2007;104:11298-303.
25. Chiou M, Xu Y, Longaker MT. Mitogenic and Chondrogenic Effects of Fibroblast

Growth Factor-2 in Adipose-Derived Mesenchymal Cells. Biochem Biophys Res Commun 2006;343:644-52.

26. Tabata Y, Hijikata S, Ikada Y. Enhanced Vascularization and Tissue Granulation by Basic Fibroblast Growth Factor Impregnated in Gelatin Hydrogels. J Control Release 1994;31:189-99.
27. Hausman GJ, Wright JT, Richardson RL. The Influence of Extracellular Matrix Substrata on Preadipocyte Development in Serum-Free Cultures of Stromal-Vascular Cells. J Anim Sci 1996;74:2117-28.
28. Kiwaki K, Novak CM, Hsu DK, Liu FT, Levine JA. Galectin-3 Stimulates Preadipocyte Proliferation and Is up-Regulated in Growing Adipose Tissue. Obesity (Silver Spring) 2007;15:32-9.
29. Masella R, Vari R, D'Archivio M, Santangelo C, Scazzocchio B, Maggiorella MT, Sernicola L, Titti F, Sanchez M, Di Mario U, Leto G, Giovannini C. Oxidised LDL Modulate Adipogenesis in 3T3-L1 Preadipocytes by Affecting the Balance between Cell Proliferation and Differentiation. FEBS Lett 2006;580:2421-9.
30. Roncari DA, Van RL. Promotion of Human Adipocyte Precursor Replication by 17beta-Estradiol in Culture. J Clin Invest 1978;62:503-8.
31. Hutley LJ, Herington AC, Shurety W, Cheung C, Vesey DA, Cameron DP, Prins JB. Human Adipose Tissue Endothelial Cells Promote Preadipocyte Proliferation. Am J Physiol Endocrinol Metab 2001;281:E1037-44.
32. Anderson LA, McTernan PG, Barnett AH, Kumar S. The Effects of Androgens and Estrogens on Preadipocyte Proliferation in Human Adipose Tissue: Influence of Gender and Site. J Clin Endocrinol Metab 2001;86:5045-51.

33. Gregoire FM, Smas CM, Sul HS. Understanding Adipocyte Differentiation. Physiol Rev 1998;78:783-809.
34. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell Shape, Cytoskeletal Tension, and Rhoa Regulate Stem Cell Lineage Commitment. Dev Cell 2004;6:483-95.
35. Tajima R, Kawaguchi N, Horino Y, Takahashi Y, Toriyama K, Inou K, Torii S, Kitagawa Y. Hypoxic Enhancement of Type IV Collagen Secretion Accelerates Adipose Conversion of 3T3-L1 Fibroblasts. Biochim Biophys Acta 2001;1540:179-87.
36. Flynn LE, Prestwich GD, Semple JL, Woodhouse KA. Proliferation and Differentiation of Adipose-Derived Stem Cells on Naturally Derived Scaffolds. Biomaterials 2008;29:1862-71.

PART II

REGENERATION OF VARIOUS TISSUES BY CELL SCAFFOLD AND RELEASE MATERIALS OF BIO-SIGNALING MOLECULE WITHOUT CELLS

Chapter 4

De novo regeneration of adipose tissue by matrigel of natural scaffold and gelatin microspheres containing basic fibroblast growth factor

INTRODUCTION

In plastic and reconstructive surgery, for augmentation of lost soft tissues [1], transplantation of autologous fat grafts of a few millimeters size and semiliquid has been sometimes performed for depressed regions or scars in the breast and facial areas [2, 3]. However, progressive absorption of the tissue graft was observed with time, while cells in free fat autografts were hardly proliferated and necrotic adipocytes were often replaced by host fibrous tissue in most areas [4-8].

It has been recognized in the recent cell biology that adipocyte lineage is derived from multipotential mesenchymal stem cells with differentiation capacity [9]. These stem cells are morphologically and biochemically converted to mature adipocytes (adipose cells) by way of adipose precursor cells. Among the precursor cells are preadipocytes that have committed or determined to become fat cells and are included in interstitial cells having fibroblast-like morphology [10].

Recently, tissue engineering has been used as a new biomedical technology to repair or regenerate a body defect by taking advantage of cells, the artificial matrix

(scaffold) for cell proliferation and differentiation, and growth factor [11]. Basically, there are two research strategies of tissue engineering to induce tissue regeneration. The first strategy is to use cells that proliferate and differentiate to regenerate tissue. The cells are brought into a body site where regeneration of tissue is expected. The second way is to induce *in vivo* tissue regeneration based on precursor or stem cells, such as preadipocytes, originally existing in the body. If it is possible to provide a microenvironment suitable for the proliferation and differentiation of such cells, *de novo* tissue regeneration will be expected without exogenous transplantation of key cells for regeneration. Recently, Kawaguchi *et al.* have demonstrated that *de novo* adipogenesis in the mouse subcutis could be achieved only by injection of the simple mixture of bFGF and an extract of basement membrane extract (Matrigel) [12] of natural scaffold. Mixing with Matrigel enabled bFGF to promote the angiogenic response [13], which is essential for generation and maintenance of the adipose tissue. Tabata *et al.* have demonstrated that an enhanced angiogenic effect was achieved through controlled release of bFGF from gelatin hydrogels [14-18]. Following subcutaneous implantation of Matrigel combined with gelatin microspheres containing bFGF into the mouse back, significantly higher *de novo* adipogenesis at the implanted site was observed than implantation of the mixture of Matrigel and bFGF solution [19]. These findings experimentally support that it was possible to induce adipogenesis by creating an environment suitable to tissue regeneration in the body.

The objective of this chapter is to investigate the time profile of *de novo* regeneration of adipose tissue in Matrigel of natural scaffold by gelatin microspheres for bFGF release. Following subcutaneous implantation of Matrigel mixed with the gelatin

microspheres containing bFGF into the back of mice, *de novo* regeneration of adipose tissue was histologically evaluated at different time intervals from the viewpoint of volume occupied by adipose tissue formed and compared with that of Matrigel mixed with bFGF in the solution form. The number of cells infiltrated into Matrigel by co-implantation with gelatin microspheres containing bFGF was examined.

EXPERIMENTAL

Materials

An aqueous solution of human recombinant bFGF with an isoelectric point (IEP) of 9.6 (10 mg/ml) was kindly supplied by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). A gelatin sample with an IEP of 5.0 (Nitta Gelatin Co., Osaka, Japan) was prepared through an alkaline process of type I collagen obtained from bovine bone. It is found that the “acidic” gelatin complexes with the “basic” bFGF mainly due to their electrostatic interaction [20, 21]. The Matrigel used here was Matrigel® basement membrane matrix of growth factor reduced type (Lot no. 911947; Becton Dickinson Labware, Bedford, MA) prepared to minimize the effect of growth factor as much as possible. Glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification.

Preparation of gelatin microspheres containing bFGF

Gelatin microspheres were prepared through GA crosslinking of gelatin

aqueous solution in an emulsion state, as described in Chapter 1. The water content of the gelatin microspheres was 95 %, when calculated from the microsphere volume before and after swelling in phosphate-buffered saline solution (PBS, pH 7.4) for 24 hr at 37 °C.

The original bFGF solution was diluted with DDW to adjust the solution concentration of 10 μg/ml. The aqueous solution of bFGF (10 μl) was dropped onto 2 mg of freeze-dried gelatin microspheres, followed by leaving at 25 °C for 1 hr for impregnation of bFGF into the microspheres. The bFGF solution was completely absorbed into the microspheres through the impregnation process because the solution volume was less than that theoretically required for the equilibrated swelling of microspheres. The bFGF amount was selected since it was the most effective dose to induce *de novo* adipogenesis previously reported [19]. An animal experiment revealed that the gelatin microspheres used were degraded with time in the back subcutis of mice to completely disappear 3 weeks later.

***In vivo* experiments**

The gelatin microspheres (2 mg) swollen with the bFGF aqueous solution were mixed with 100 μl of Matrigel precooled on ice. The mixture was left for 1 hr at 37 °C to allow Matrigel to form a hydrogel. As control, 10 μl of aqueous solutions containing 0.1 μg of bFGF was similarly mixed with Matrigel.

Under anesthesia, the mixture of Matrigel with gelatin microspheres containing bFGF, free bFGF, or bFGF-free PBS was carefully implanted into the back subcutis of female BALB/c mice, 6 weeks old (Shimizu Laboratory Supply, Kyoto, Japan) 1.5 cm

from the tail root at the body center where it is free of originally existing adipose tissue. Each experimental group at every sampling point was composed of five mice. At 2, 4, 6, 12, and 15 weeks after implantation, the mice were sacrificed by an overdose injection of anesthetic, and the skin, including the implanted site (2 x 2 cm^2), was carefully taken off for the subsequent biological examinations. Photographs of the skin flaps were taken to view tissue appearance around the implanted site of Matrigel.

De novo regeneration of adipose tissue at the site implanted was assessed from the viewpoint of histology, the volume occupied by adipocytes regenerated. All the skin flaps were fixed with 10% neutralized formalin solution, embedded in paraffin, and sectioned (4 μm thickness) at the portion of implanted site as central as possible, followed by staining with hematoxylin and eosin (HE). Microphotographs of five cross sections from five different mice were taken at a similar magnification to histologically evaluate the *de novo* regeneration of adipose tissue and angiogenesis. The same area of interest (three portions/cross-section, 0.8 x 0.5 mm^2) was randomly selected, and the area ratio of matured adipocytes occupied to the Matrigel implanted for every portion was measured to evaluate the occupied percentage of adipose tissue. The cross sectional shape of tissue regenerated in Matrigel was generally ellipsoid, and from all the five histological sections for each experimental group, the maximum thickness of tissue formed was measured, while the length of long and short axes of tissues regenerated was measured from the skin flap photographs. The volume of tissue formed was calculated from the thickness and lengths measured on the assumption that the shape of tissue regenerated is of an ellipsoidal sphere. The occupied percentage was multiplied by the volume of tissue regenerated to obtain the volume of adipose tissue regenerated.

Statistical analysis

All the data were analyzed by Fisher's LSD test for multiple comparison and the statistical significance was accepted at $p < 0.05$. Experimental results were expressed as the mean 6 standard deviation of the mean (SD).

RESULTS

***De novo* regeneration of adipose tissue and vascularization by Matrigel and gelatin microspheres containing bFGF or free bFGF**

Figure 1 shows the tissue appearance of mouse subcutis 4, 6, 12, and 15 weeks after implantation of PBS, free bFGF, and gelatin microspheres containing bFGF together with Matrigel. When Matrigel was co-implanted with gelatin microspheres containing bFGF, new formation of tissue mass was found at the site implanted with implantation time, while many blood vessels were distributed in the tissue formed. The similar change in tissue appearance was observed at the Matrigel implanted with free bFGF although the regeneration of blood vessels was less. Upon implanting with neither gelatin microspheres containing bFGF nor free bFGF, appearance of the implanted Matrigel did not change very much, irrespective of the implantation time.

Figure 2 shows the histological sections at the site implanted of Matrigel different time periods after implantation. Apparently, co-implantation of bFGF regenerated *de novo* adipose tissue in Matrigel with implantation time. Matured adipocytes accumulating lipid inside were observed in the tissue mass formed 4 weeks

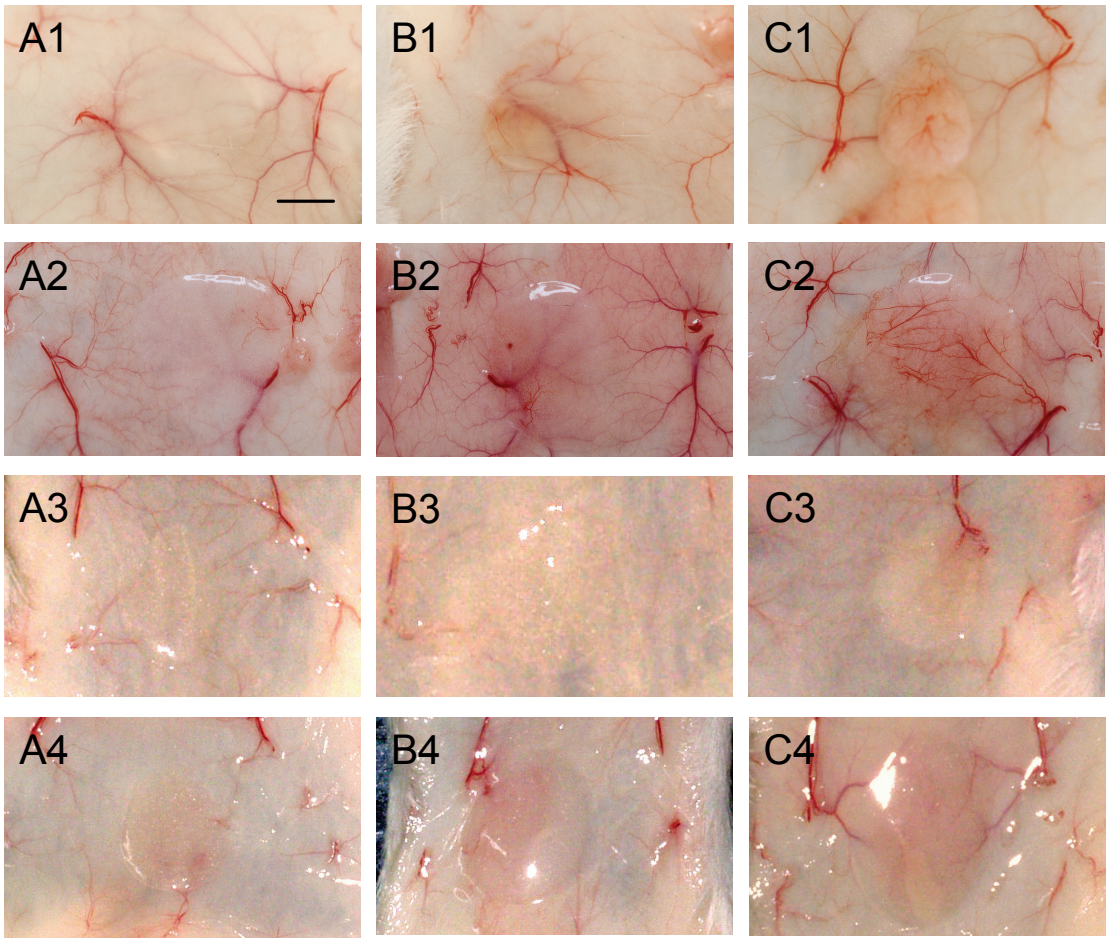

Figure 1. Tissue appearance of mouse subcutis at different time periods after implantation of Matrigel mixed with PBS (A), 0.1 μg of bFGF (B), and gelatin microspheres containing 0.1 μg of bFGF (C). Observation was done at 4 (A1, B1, C1), 6 (A2, B2, C2), 12 (A3, B3, C3), and 15 weeks (A4, B4, C4) after implantation. Scale bar = 3 mm.

after implantation of Matrigel combined with gelatin microspheres containing bFGF, whereas Matrigel combined free bFGF was not effective. There was a layer of original adipose tissue below the dermis and above the subcutaneous fascia layer. However, the histological site of *de novo* regeneration of adipose tissue was different from that of the

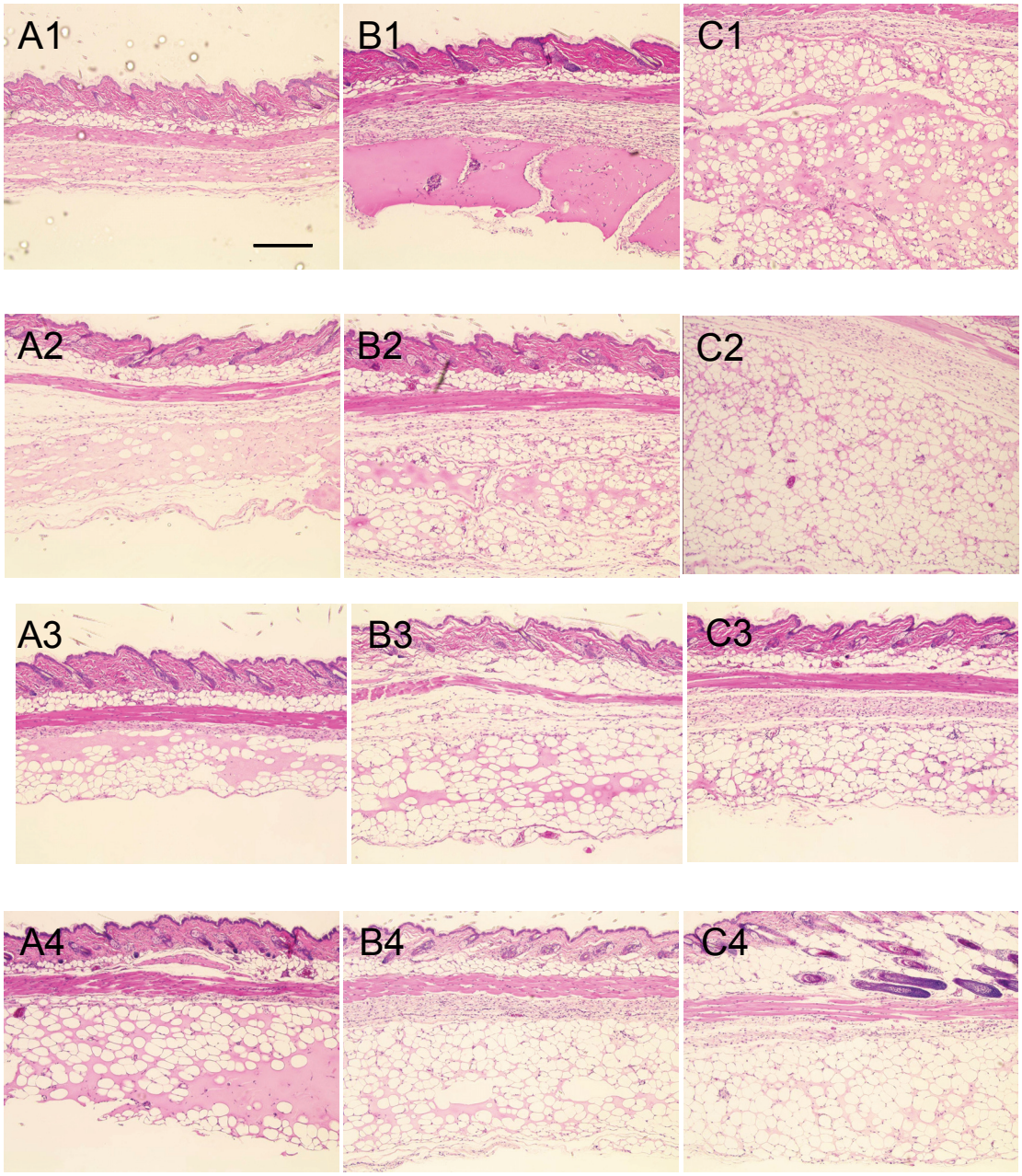

Figure 2. Histological sections of mouse subcutis at different time periods after implantation of Matrigel mixed with PBS (A), 0.1 μg of bFGF (B), and gelatin microspheres containing 0.1 μg of bFGF (C). Observation was done at 4 (A1, B1, C1), 6 (A2, B2, C2), 12 (A3, B3, C3), and 15 weeks (A4, B4, C4) after implantation. (D) is a histological section of mouse subcutis at the implantation site. (HE staining, Scale bar = 300 μm)

original adipose tissue. Delayed regeneration of adipose tissue and less number of matured adipocytes were detected in the tissue mass formed by implantation of Matrigel plus PBS.

Time course of volume occupied by adipose tissues regenerated by Matrigel and gelatin microspheres containing bFGF or free bFGF

Figure 3 shows change in the volume of adipose tissue formed by Matrigel combined with gelatin microspheres containing bFGF or free bFGF. bFGF combination was significantly effective in increasing the volume of adipose tissue regenerated with implantation time, in contrast to Matrigel alone. However, the increasing extent in the tissue volume was faster for the mixed Matrigel and gelatin microspheres than the mixed Matrigel and free bFGF. The adipose tissue formed 4 weeks after implantation was significantly large in volume, and the enlarged volume was retained until 15 weeks. Matrigel plus PBS did not contribute to any enhancement of the tissue volume during the initial implantation periods, although delayed enlargement of tissue volume was observed.

DISCUSSION

Tissue engineering has been expected to be a biomedical form for regenerative medicine. The basic idea is that tissue regeneration is realized by using cells and providing an environment suitable for their proliferation and differentiation. For adipose

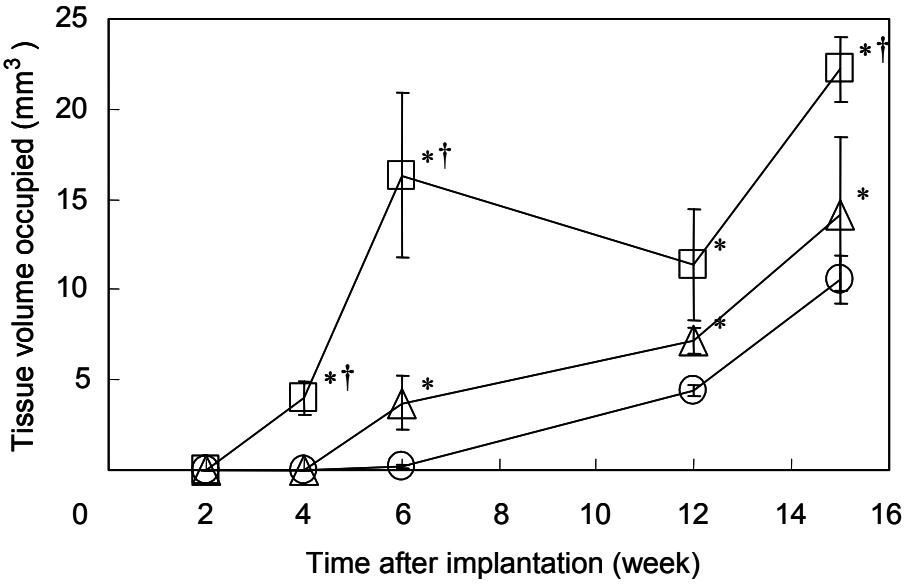

Figure 3. Time course of volume occupied by adipose tissue regenerated at different time periods after implantation of Matrigel mixed with PBS (○), 0.1 μg of bFGF (△), and gelatin microspheres containing 0.1 μg of bFGF (□). *: $p < 0.05$ significant against the group implanted with Matrigel mixed with PBS at the corresponding time; †: $p < 0.05$ significant against the group implanted with Matrigel mixed with free bFGF at the corresponding time.

tissue engineering, the precursor of adipocytes is one of the cells. It has been demonstrated in a rodent system that adipose precursor cells possess the potential ability to generate new adipose tissues [22]. The fat depots of mice express a large amount of early markers of adipocytes differentiation [23]. A large population of stromal-vascular cells from subcutaneous fat tissues of elderly men and women have the potential to differentiate *in vitro* into adipocytes [24]. These findings clearly indicate that proliferation and differentiation of adipose precursor cells can be promoted if an environment for the cell-based tissue regeneration can be created in the body.

Considering cellularity in the adipose tissue, the number of adipocytes and

their precursor cells is less than one-half of total cell number in the tissue; the remaining cells are various blood cells, endothelial cells, and pericytes. It is well recognized that this type of cell develops a vascular supply essential for the generation and maintenance of adipose tissue. There is no doubt that angiogenesis will be one of the environments that allow adipose precursor cells to proliferate and differentiate into matured adipocytes. Indeed, the previous study indicated that such an environment could be artificially created by implantation of Matrigel together with the release system of bFGF [19]. It is possible that angiogenesis induced by the controlled release of bFGF results in the promoted migration of adipose precursor cells into the vascularized Matrigel and the subsequent proliferation and maturation. The other possible contribution is a direct adipogenic effect of bFGF. Sheep preadipocytes have been reported to differentiate in a culture medium containing bFGF [25]. It may be that bFGF accelerates the proliferation of preadipocytes as well as adipocyte differentiation, resulting in enhanced *de novo* regeneration of adipose tissue.

In addition to the bFGF-induced angiogenesis, Matrigel is an essential material to induce *de novo* regeneration of adipose tissue. A basement membrane was prepared through a detergent treatment of mouse muscles and the membrane fragment was mixed with the gelatin microspheres containing bFGF. When the mixture was placed into the sponge of collagen type I as a scaffold and implanted into the back subcutis of mice, *de novo* regeneration of adipose tissue was achieved at the implanted site of sponge [11]. This suggests that the basement membrane contained the substances or components which enable the precursor cells to proliferate and differentiate for adipogenesis. It is reported that Matrigel enhanced attachment and spreading of preadipocytes [26]. In

addition, it was revealed in Chapter 3 that the coating of mixed basement membrane components and type I collagen promoted proliferation and adipogenic differentiation of human preadipocytes. It cannot be ruled out the possibility that Matrigel has a function as the scaffold to efficiently promote the cell proliferation and differentiation of adipose precursor cells.

One possible way to artificially enhance *in vivo* angiogenesis is to allow bFGF to controlled release over an extended period of time. Significant angiogenesis was demonstrated to induce through controlled release of biologically active bFGF from gelatin hydrogel microspheres, in marked contrast to bFGF administered in the solution form [14-17]. Histological examinations revealed that mixing the gelatin microspheres incorporated bFGF induced angiogenesis in Matrigel to a greater extent than free bFGF mixing (Figure 2). It is reported that Matrigel functions as the carrier for bFGF release. However, Matrigel itself functions to induce angiogenic activity of bFGF to some extent [13], but the efficacy is not as high as that of the gelatin microspheres. The initially enhanced adipogenesis by co-implantation of gelatin microspheres containing bFGF may be caused by this release nature more controllably than the Matrigel itself. There must exist an optimal concentration range and maintenance period of bFGF for adipogenesis. It is conceivable that the controlled release by gelatin microspheres enables bFGF to create these conditions in Matrigel, resulting in accelerated adipogenesis (Figure 2). The period of bFGF release was about 3 weeks from the microspheres, while the increase in the volume of adipose tissue regenerated was observed between 4 and 6 weeks. This time lag may be explained based on the biological action of bFGF. bFGF mainly acts on preadipocytes to accelerate their *in vivo*

proliferation. However, it has little influence on their differentiation. As a result, it is possible that the bFGF release would enable preadipocytes to increase their number. Thereafter, it needs a few weeks to differentiate the increased preadipocytes into matured fat cells *in vivo*.

When the number of cells infiltrated into Matrigel was assessed by counting the number of cell nuclei from the histological section of every implanted sample, the gelatin microspheres containing bFGF enhanced the number of infiltrated cells to a significantly high extent compared with in contrast to free bFGF, empty gelatin microspheres, and PBS (data not shown). This finding indicates that the bFGF release more or less affected the cell recruitment. However, in this study, as the cell type is not differentiated, one cannot differentiate between an increase in adipogenesis and an increase in wound healing or foreign body response.

Faster increase and long retention in the volume of adipose tissue regenerated were achieved by co-implantation of gelatin microspheres containing bFGF (Figure 3). These findings demonstrate superiority of mixed Matrigel and gelatin microspheres containing bFGF in *de novo* adipogenesis. Some studies are presently underway to investigate the key component of Matrigel for adipogenesis promotion as well as the effect of release period of bFGF on the *de novo* adipogenesis.

REFERENCES

1. Billings E, Jr., May JW, Jr. Historical Review and Present Status of Free Fat Graft Autotransplantation in Plastic and Reconstructive Surgery. Plast Reconstr Surg 1989;83:368-81.
2. Ellenbogen R. Free Autogenous Pearl Fat Grafts in the Face--a Preliminary Report of a Rediscovered Technique. Ann Plast Surg 1986;16:179-94.
3. Hartrampf CR, Scheflan M, Black PW. Breast Reconstruction with a Transverse Abdominal Island Flap. Plast Reconstr Surg 1982;69:216-25.
4. Ersek RA. Transplantation of Purified Autologous Fat: A 3-Year Follow-up Is Disappointing. Plast Reconstr Surg 1991;87:219-27; discussion 28.
5. Fagrell D, Enestrom S, Berggren A, Kniola B. Fat Cylinder Transplantation: An Experimental Comparative Study of Three Different Kinds of Fat Transplants. Plast Reconstr Surg 1996;98:90-6; discussion 7-8.
6. Peer LA. The Neglected Free Fat Graft. Plast Reconstr Surg 1956;18:233-50.
7. Smahel J. Failure of Adipose Tissue to Heal in the Capsule Preformed by a Silicone Implant. Eur J Plast Surg 1985;8:109-15.
8. Smahel J. Experimental Implantation of Adipose Tissue Fragments. Br J Plast Surg 1989;42:207-11.
9. Caplan AI. Mesenchymal Stem Cells. J Orthop Res 1991;9:641-50.
10. Ailhaud G, Grimaldi P, Negrel R. Cellular and Molecular Aspects of Adipose Tissue Development. Annu Rev Nutr 1992;12:207-33.
11. Tabata Y. The Importance of Drug Delivery Systems in Tissue Engineering.

Pharmaceutical Science and Technology Today 2000;3:80-9.

12. Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De Novo Adipogenesis in Mice at the Site of Injection of Basement Membrane and Basic Fibroblast Growth Factor. Proc Natl Acad Sci U S A 1998;95:1062-6.
13. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR. A Simple, Quantitative Method for Assessing Angiogenesis and Antiangiogenic Agents Using Reconstituted Basement Membrane, Heparin, and Fibroblast Growth Factor. Lab Invest 1992;67:519-28.
14. Ikada Y, Tabata Y. Protein Release from Gelatin Matrices. Adv Drug Deliv Rev 1998;31:287-301.
15. Tabata Y, Hijikata S, Ikada Y. Enhanced Vascularization and Tissue Granulation by Basic Fibroblast Growth Factor Impregnated in Gelatin Hydrogels. J Control Release 1994;31:189-99.
16. Tabata Y, Hijikata S, Muniruzzaman M, Ikada Y. Neovascularization Effect of Biodegradable Gelatin Microspheres Incorporating Basic Fibroblast Growth Factor. J Biomater Sci Polym Ed 1999;10:79-94.
17. Tabata Y, Ikada Y. Potentiated in Vivo Biological Activity of Basic Fibroblast Growth Factor by Incorporation Intopolymer Hydrogel Microsphere. 4th Japan International SAMPE Symposium 1995;4:577-82.
18. Tabata Y, Nagano A, Ikada Y. Biodegradation of Hydrogel Carrier Incorporating Fibroblast Growth Factor. Tissue Eng 1999;5:127-38.
19. Tabata Y, Miyao M, Inamoto T, Ishii T, Hirano Y, Yamaoki Y, Ikada Y. De Novo Formation of Adipose Tissue by Controlled Release of Basic Fibroblast Growth

Factor. Tissue Eng 2000;6:279-89.

20. Muniruzzaman M, Tabata Y, Ikada Y. Physicochemical Properties of Basic Fibroblast Growth Factor-Gelatin Complex. Journal of Bioactive and Compatible Polymers 2000;15:365-75.
21. Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In Vitro Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials 1998;19:1781-9.
22. Green H, Kehinde O. Formation of Normally Differentiated Subcutaneous Fat Pads by an Established Preadipose Cell Line. J Cell Physiol 1979;101:169-71.
23. Ailhaud G. Extracellular Factors, Signalling Pathways and Differentiation of Adipose Precursor Cells. Curr Opin Cell Biol 1990;2:1043-9.
24. Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF. Promoting Effect of Glucocorticoids on the Differentiation of Human Adipocyte Precursor Cells Cultured in a Chemically Defined Medium. J Clin Invest 1989;84:1663-70.
25. Broad TE, Ham RG. Growth and Adipose Differentiation of Sheep Preadipocyte Fibroblasts in Serum-Free Medium. Eur J Biochem 1983;135:33-9.
26. Hauner H, Rohrig K, Petruschke T. Effects of Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF) and Fibroblast Growth Factor (FGF) on Human Adipocyte Development and Function. Eur J Clin Invest 1995;25:90-6.

Chapter 5

In situ regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor

INTRODUCTION

Breast cancer is exemplified as one of the most common tumors for women in the western world. Mastectomy results in the loss of one or both breasts, which has often caused mental problems for patients. Therefore, reconstruction trials of female breasts have been performed and reported [1-5]. Autografting of fat pad has a long history in plastic and reconstructive surgeries for augmentation of soft tissues [6]. It was reported that autologous adipose tissues, such as the fat graft of a few millimeters in size and semiliquid, were transplanted to depressed regions or scars in the breast [1, 7]. Despite the enthusiasm for such free-fat autografting, however, researchers have been disappointed by the progressive absorption over time of grafted tissue [8-12]. Microscopic examination of free-fat autografts removed demonstrated the necrosis of adipocytes and occupation of graft tissue by the host. In addition, the transplanted fat cells hardly proliferated. On the other hand, artificial implants, such as silicone or saline prosthesis, have been clinically used. However, all of the procedures have substantial disadvantages and morbidity [4]. Recently, tissue engineering has been gaining favor as

a newly emerging biomedical technology to repair or regenerate a body defect by combining cells of high proliferation and differentiation potential with an artificial matrix of cells scaffold and growth factor [13]. This tissue engineering technology is also applicable for regeneration of adipose tissue, and some trials of adipose tissue engineering have been reported [14-17].

Preadipocytes, which are defined as a precursor of adipocytes present between mature adipocytes in adipose tissue, were expected as one cell source potential for soft-tissue engineering [11]. The proliferative activity of preadipocytes is high, whereas matured adipocytes lose their capacity to divide [18]. The adipocyte precursor cells have been isolated from the stroma of adult adipose tissue, while their differentiation in culture has been investigated in the last two decades [19]. This has made it possible to isolate the precursor cells from the processed lipoaspirate from patients, and the cells differentiate into osteogenic, adipogenic, chondrogenic, and neurogenic lineages, which is advantageous for tissue engineering [17, 20]. There are two possible strategies based on tissue engineering to induce regeneration of adipose tissue. The first strategy is to make use of cells that have the potential for proliferation and differentiation to regenerate adipose tissue. The cells are brought into a body site where regeneration of adipose tissues is expected. Adipose tissue engineering by use of collagen scaffold combined with human preadipocytes has been reported [21, 22]. This is the first report of regeneration of adipose tissue by use of human cells, which experimentally confirmed the possibility of adipose tissue engineering for human trial. If originally existing preadipocytes are used for *in situ* regeneration of adipose tissue, it will be more convenient because it takes much time to isolate and proliferate the cells. Thus, the

second strategy is to induce *in vivo* regeneration of adipose tissue based on preadipocytes originally existing in the body. If it is possible to provide a local environment suitable for the proliferation and differentiation of such cells, formation of adipose tissue will be expected without exogenous transplantation of cells necessary for adipogenesis. It has been demonstrated that adipogenesis in the mouse subcutis could be achieved only by injection of simple mixtures of basic fibroblast growth factor (bFGF) and an extract of basement membrane extract (Matrigel®) [23]. Tabata *et al.* have demonstrated that controlled release from gelatin hydrogels enabled bFGF to significantly enhance the angiogenic effect *in vivo* [24-28]. After subcutaneous implantation of Matrigel® combined with this bFGF release system into the mouse back, significantly higher adipogenesis at the implanted site was observed than that of the mixed Matrigel® and free bFGF in Chapter 4. These findings experimentally confirmed that it was possible to induce adipogenesis even by preadipocytes originally present if a local environmental field suitable to tissue regeneration in the body is provided. However, because the Matrigel® scaffold is a mouse-derived material, the human application is practically impossible. Thus, it has been tried to create the regeneration environment by combining a sponge scaffold of type I collagen and the controlled release system of bFGF. In Chapter 1, regeneration of adipose tissue by collagen scaffolds and gelatin microspheres containing basic fibroblast growth factor combined with human preadipocytes was observed within 6 weeks postoperatively. Subcutaneous implantation of a collagen scaffold incorporating the release system of bFGF without human preadipocytes did not induce adipogenesis in the back of nude mice. Considering the regeneration of fat tissue after mastectomy, defect to be regenerated is adipose tissue.

Thus, it is necessary to prepare an animal model in which a defect is surrounded with inherent adipose tissue.

The objective of this chapter is to examine regeneration of adipose tissue in a new defect model that is close to the environment of the breast defect after mastectomy. A collagen sponge scaffold was incorporated by gelatin microspheres containing bFGF for controlled release and implanted into the defect of a rat fat pad to evaluate the *in situ* formation of adipose tissue in the sponge. Regeneration of adipose tissue was evaluated by determining the percentage of adipose tissue regenerated in histological sections. The effect of the bFGF dose and the release profile of bFGF from gelatin microspheres, and the effect of preadipocytes on the regeneration of adipose tissue as well as the time course of adipogenesis were examined.

EXPERIMENTAL

Materials

An aqueous solution of type I collagen, prepared from porcine tendon with pepsin treatment (3 mg/ml, pH 3.0) in HCl was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. A polypropylene (PP) mesh with an aperture of 200 μm (Figure. 1) was purchased from FLON Industry Inc. An aqueous solution of human recombinant bFGF (10 mg/ml) was kindly supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. A gelatin sample with an isoelectric point (IEP) of 5.0 (Nitta Gelatin Inc., Osaka, Japan) was prepared through an alkaline process of type I collagen obtained from bovine bone.

Minimal essential medium alpha and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical Co., Ltd., and ICN Biomedicals, Inc., USA, respectively. Double-distilled and deionized water (DDW) was prepared with a Milli-Q water filter system (MILLI-Q SP UF: Millipore Co., MA, USA). Glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries Ltd., Kyoto, Japan and used without further purification.

Fabrication of collagen-PP scaffold

For preparation of collagen sponges, briefly, 0.1 ml of collagen solution was poured into a polystyrene mold (96 well; COSTAR, Corning Inc., NY, USA). The collagen solution was frozen at -80 °C and freeze-dried to obtain a collagen sponge. The freeze-dried sponge was punched out to obtain collagen sponge discs (5.5 mm in diameter, 3.0 mm thickness). The sponge discs were dehydrothermally cross-linked at 140 °C for 12 hr under vacuum condition (0.1 Torr), followed by chemical crosslinking in 0.2 %(w/v) of GA aqueous solution (4 °C, 12 hr). The resulting sponge discs were immersed in 50 mM glycine aqueous solution at 37 °C for 1 hr to block the residual aldehyde groups of GA. Then, the collagen sponge discs were rinsed three times with DDW at 37 °C and freeze-dried. The PP mesh was immersed in acetone for 1 hr to remove oils and fats, and rinsed five times with DDW for 10 min. The collagen sponge disc was surrounded by the PP mesh. Because the mesh is not biodegradable and biocompatible, it is possible to recognize the boundary between the collagen sponge and the surrounding original tissue even after sponge degradation *in vivo*. The cross-linked collagen-PP scaffolds were sterilized with ethylene oxide gas at 40 °C for the following

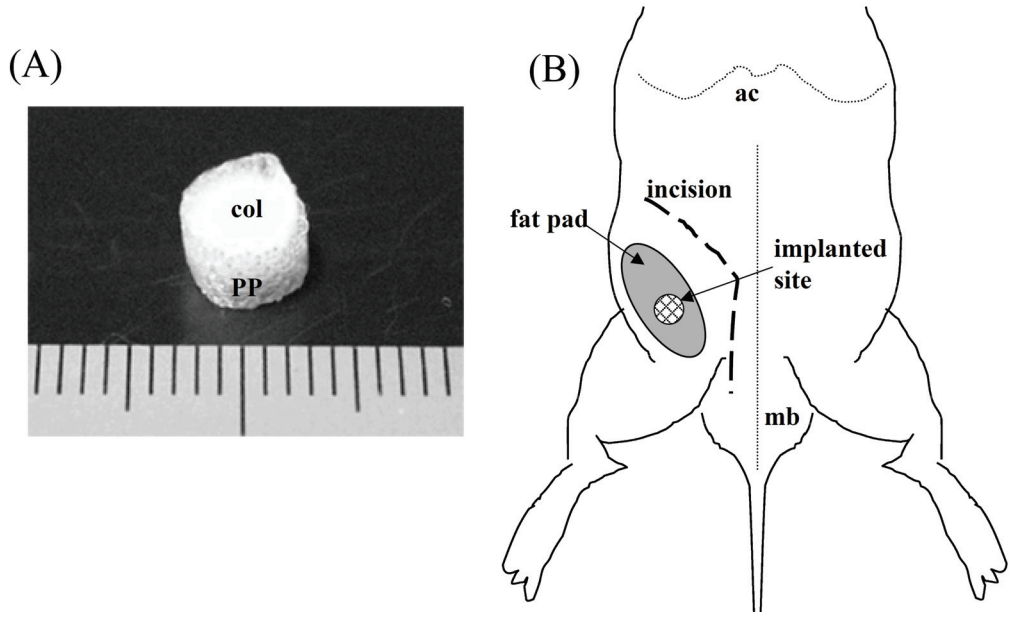

Figure 1. (A) The surrounding of a collagen sponge disc (col) was lapped by a polypropylene mesh (PP) with an aperture of 200 μm to prepare the collagen-PP scaffold. (B) Schematic illustration of *in vivo* experiment. The collagen-PP scaffold was incorporated by gelatin mirospheres containing bFGF with or without preadipocytes and implanted into the defect of rat fat pad: ac, arcus costarum and mb; medial boundary.

in vivo experiments (Figure. 1).

Preparation of gelatin microspheres containing bFGF

Gelatin microspheres were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state, described in Chapter 2. The water contents of gelatin microspheres prepared were 99, 98, 95, and 90 % when 25, 50, 100, and 500 μl of 25 %(w/v) of GA solution were added to 20 ml of crosslinking reaction solution. The original bFGF solution was diluted with DDW to adjust the bFGF concentrations to 0.5,

5, 50, and 500 μg/ml. The aqueous solution of bFGF (20 μl) was dropped onto 1 mg of freeze-dried gelatin microspheres, followed by leaving at 25 °C for 1 hr for impregnation of bFGF into the microspheres. As a control, 20 μl of PBS was dropped onto 1 mg of freeze-dried gelatin microspheres to prepare bFGF-free, empty gelatin microspheres.

Isolation and culture of rat preadipocytes

Preadipocytes were primarily isolated from the fat pad of syngeneic Wistar rats (Shimizu Laboratory Supply, Japan). The fat pad was washed with phosphate-buffered saline solution (PBS, pH 7.4) to carefully remove blood cells, then minced and digested by 520 U/ml collagenase (Nitta Gelatin Inc., Osaka, Japan) in a water bath at 37 °C for 60 min with shaking. The digested fat pad was suspended in Medium 199 containing 10 % FBS, followed by centrifugation (200 x g, 5 min, 4 °C) to collect the supernatant. After washing twice with the medium, the cells obtained were cultured in a cell-culture flask (75 cm^2, Corning 430720, 1 x 10^3 cells/cm^2) in the medium containing 0.1 μg/ml of bFGF at 37 °C and 5 % CO_2- 95 % air atmosphere pressure. The cells were expanded by subculturing two times and subjected to *in vivo* experiments. The cell morphology was fibroblast-like. When cultured in the presence of 50 nM of insulin, 100 nM of dexamethasone, 10 μg/ml of transferrin, and 200 pM of triiodothyronine for 14 days, the cells accumulated fat droplets inside. This suggested that the isolated cells had an inherent nature to differentiate into matured adipocytes.

In vivo experiments

The *in vivo* experiments are listed in Table 1. Groups I and V were performed to evaluate the effect of the bFGF concentration, Group I (1.0) was used to evaluate the effect of the implantation time period, Groups I (1.0), II, III, and V were used to evaluate the effect of preadipocytes presence, and Groups IV and VI were to evaluate the bFGF release profile on the *in situ* formation of adipose tissue in the collagen-PP scaffold. Female Wistar rats (110–140 g; Shimizu Laboratory Supply, Japan) were used. Anesthesia was achieved with a mixture of ketamine hydrochloride (40 mg/kg intraperitoneally) and xylazine (8 mg/kg intraperitoneally) with additional doses given intraperitoneally as necessary during the experiments. All the surgical procedures were done under clean conditions using steam-sterilized instruments with the surgeon masked and using laboratory coat and sterile gloves. Under anesthesia, the area around the incision was shaved and a 0.5% iodine solution was applied. In each rat, an approximately 4-cm incision was made over the skin obliquely to the median line at 10 mm cephalad to the fat pad of the rat inguinal region. The skin above the fat pad was gently reflected by the forceps to implant the collagen-PP scaffold. Then an approximately 6 mm incision was made in the fat pad, and the incision was opened in a circle. The collagen-PP scaffold incorporating gelatin microspheres containing different amounts of bFGF with or without rat preadipocytes was carefully implanted into the circle of the fat pad of the rat as the side of the collagen-PP scaffold was surrounded by the original adipose tissue. Also, the upper and lower surfaces of the collagen-PP scaffold faced the skin and loose areolar tissue and abdominal wall musculature. The PP mesh of collagen-PP scaffold was fixed to the original adipose tissue of rats with

Table 1. Group I: Collagen-PP scaffold incorporating gelatin microspheres containing four different amount of bFGF (μg per 1 mg of gelatin microspheres) (Group I [0.01 μg], group I [0.1], group I [1.0], and group I [10]). Group II: Collagen-PP scaffold incorporating 98 % water content gelatin microspheres containing 1 μg bFGF and 1 x 10^5 rat preadipocytes. Group III: Collagen-PP scaffold incorporating gelatin microspheres containing 20 μl of PBS and 1 x 10^5 rat preadipocytes. Group IV: Collagen-PP scaffold incorporating four different water content gelatin microspheres containing 1 μg bFGF (percent of water content) (Group IV [90], Group IV [95], Group IV [98], and Group IV [99]). Group V: Collagen-PP scaffold incorporating gelatin microspheres containing 20 μg of PBS. Group VI: Collagen-PP scaffold incorporating 1 μg bFGF without gelatin micrsphere.

Group code	bFGF (μg/site)	Water content of gelatin microspheres (%)	Preadipocytes (x 10^5 cells / site)
I (0.01)	0.01	98	0
I (0.1)	0.1	98	0
I (1.0)	1.0	98	0
I (10)	10	98	0
II	1.0	98	1
III	0	98	1
IV (90)	1.0	90	0
IV (95)	1.0	95	0
IV (98)	1.0	98	0
IV (99)	1.0	99	0
V (PBS)	0	98	0
VI (free bFGF)	1.0	-	0

nonabsorbable 5–0 sutures (Johnson & Johnson Co., New Brunswick, NJ, USA). After implantation of the collagen-PP scaffold, the skin was closed with 5–0 sutures (Johnson & Johnson Co.). Each experimental group was composed of four rats. Two, 4, and 6 weeks after implantation, the rats were sacrificed by an overdose injection of anesthetic,

and the scaffold, including the native adipose tissue, was carefully taken off. The specimen was fixed with 10 % neutralized formalin solution, embedded in paraffin, and sectioned (4 μm in thickness) at the portion of the implanted site as centrally as possible, followed by staining with hematoxylin and eosin (HE). Microphotographs of cross-sections from four different scaffolds were taken at a similar magnification to histologically evaluate the formation of adipose tissue. The percent of regenerated adipocytes was analyzed by measuring the area of matured adipocytes in the PP mesh using a computer program of Image-Pro Plus 3.01 (Media-Cybernetics, Silver Spring, MD).

Statistical analysis

All the results were statistically analyzed by the unpaired Student's t test. $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean $\pm$ the standard deviation of the mean (SD).

RESULTS

Tissue appearance of collagen-PP scaffold implanted

Figure 2 shows the tissue appearance of collagen-PP scaffold immediately and 2 weeks after implantation into the defect of fat pad. Angiogenesis was observed on the surface of collagen scaffold. The shape of scaffold was kept constant from the beginning to 2 weeks.

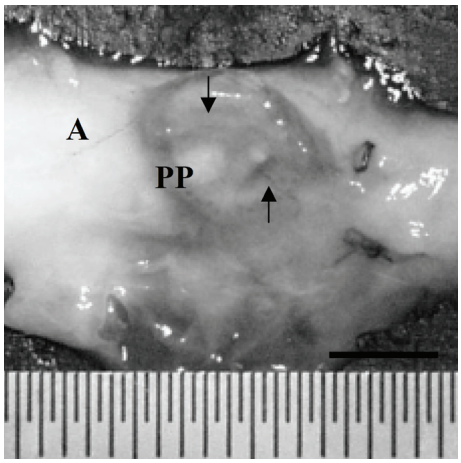

Figure 2. Tissue appearance of collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF 2 weeks after implantation into the defect of rat fat tissues. PP: polypropylene mesh. A: original adipose tissue. Arrows indicate blood vessels induced. Scale bar = 5 mm.

Influence of bFGF dose on the *in situ* regeneration of adipose tissue

Figure 3 shows the histological sections of rat fat pad 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing different amounts of bFGF. Apparently, co-implantation of microspheres containing bFGF regenerated adipose tissue in the scaffold surrounded by rat fat tissue. When the bFGF dose was 1.0 μg, gelatin microspheres containing bFGF significantly induced regeneration of adipose tissue. Less regeneration of adipose tissue was observed at bFGF doses of 0.01, 0.1, and 10 μg. Especially, 10 μg of bFGF induced an inflammatory reaction in the collagen scaffold implanted site. No regeneration of adipose tissue was observed at the collagen scaffold implanted site together with PBS or bFGF-free, empty microspheres. Figure 4 shows the bFGF dose dependence of *in situ* regeneration of adipose tissue. The regeneration was assessed by determining the area

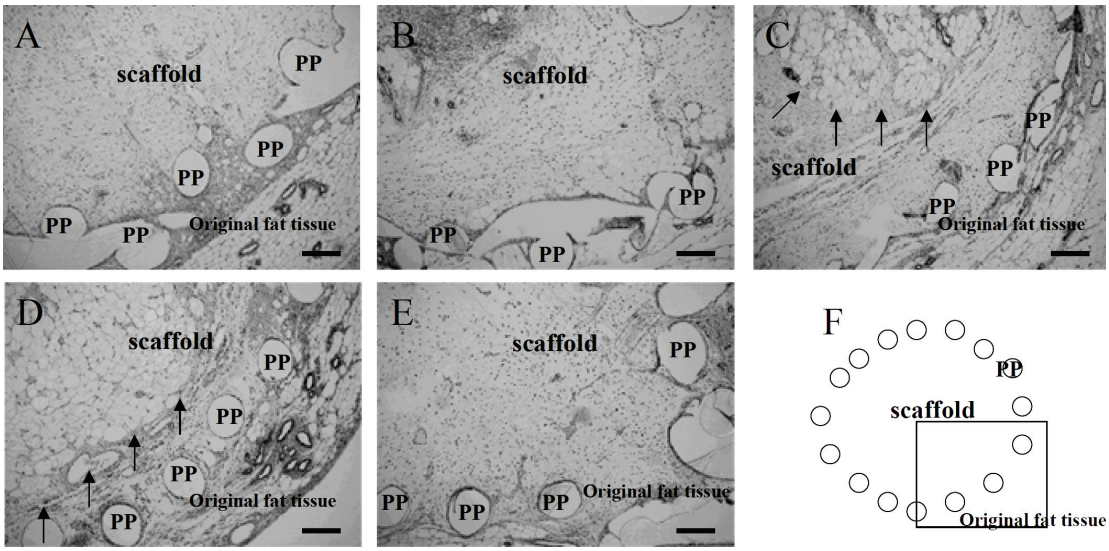

Figure 3. Histolgical sections of the site implanted of collagen-PP scaffold incorporating gelatin microspheres containing 0 (A), 0.01 (B), 0.1 (C), 1.0 (D), and 10 μg of bFGF (E) 6 weeks post-operatively. PP: polypropylene mesh. Arrows indicate adipose tissue regenerated. F schematically illustrates the area of histological section photographed. (HE staining, Scale bar = 200 μm)

percentage of adipose tissue to the total area within the PP mesh on the histological sections 4 weeks after co-implantation of collagen scaffold and gelatin microspheres containing bFGF. When gelatin microspheres containing 1.0 μg of bFGF were used, the percent regenerated of adipose tissue was significantly higher than that of other gelatin microspheres with or without bFGF incorporation.

Time course of adipose tissue regeneration by collagen scaffold and gelatin microspheres containing bFGF

Figure 5 shows the histological sections at the site implanted of collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF at different time

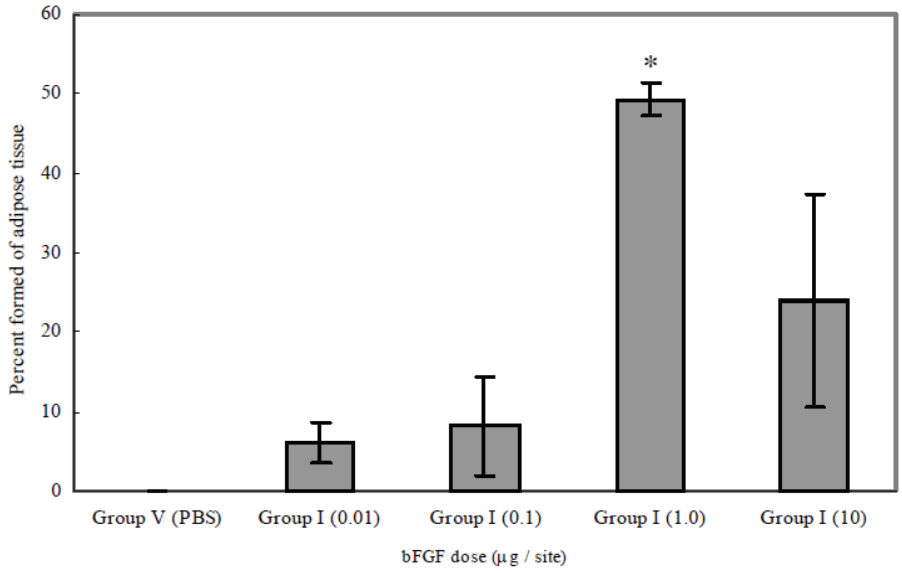

Figure 4. Effect of the bFGF dose on the percentage of adipose tissue regenerated 4 weeks after implantation of collagen-PP scaffolds incorporating gelatin microspheres containing different amounts of bFGF. *, $p<0.05$; significant against the percentage of adipose tissue regenerated at the scaffold incorporating microspheres containing bFGF at other amounts.

intervals after implantation. Mature adipocytes accumulating lipid inside were observed in the scaffold 4 weeks after implantation of collagen-PP scaffold combined with gelatin microspheres containing bFGF. However, the histological site of adipose tissue regenerated was different from that of the original adipose tissue. Figure 6 shows the time profile of area percentage of adipose tissue regenerated to the total area within the PP mesh on histologic sections after implantation of collagen-PP scaffold and gelatin microspheres containing bFGF with implantation time. The adipose tissue was regenerated with time to attain a certain significant level 4 weeks after implantation, and the regenerated level was retained until 6 weeks. There were few adipocytes in the area

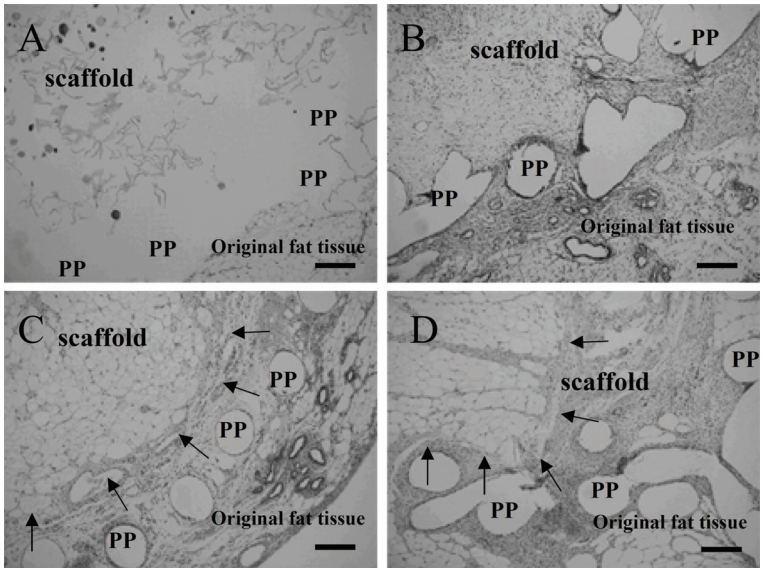

Figure 5. Histological sections of the site implanted of collagen-PP scaffold incorporating gelatin microspheres containing 1μg of bFGF 0 (A), 2 (B), 4 (C), and 6 weeks post-operatively (D). PP: polypropylene mesh. Arrows indicate adipose tissue regenerated. (HE staining, Scale bar = 200 μm)

close to the PP mesh surrounding the collagen scaffold while inflammation cells were observed between adipose tissue regenerated and the PP mesh (Figure. 5D).

Influence of preadipocytes addition on the *in situ* regeneration of adipose tissue

Figure 7 shows the histological sections of scaffold implanted 4 weeks after implantation. Matured adipocytes accumulating lipid were observed in the scaffold 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing bFGF and syngeneic rat preadipocytes. Irrespective of preadipocytes presence, a similar level of adipogenesis was observed when gelatin microspheres containing bFGF were used. However, the collagen-PP scaffold incorporating rat

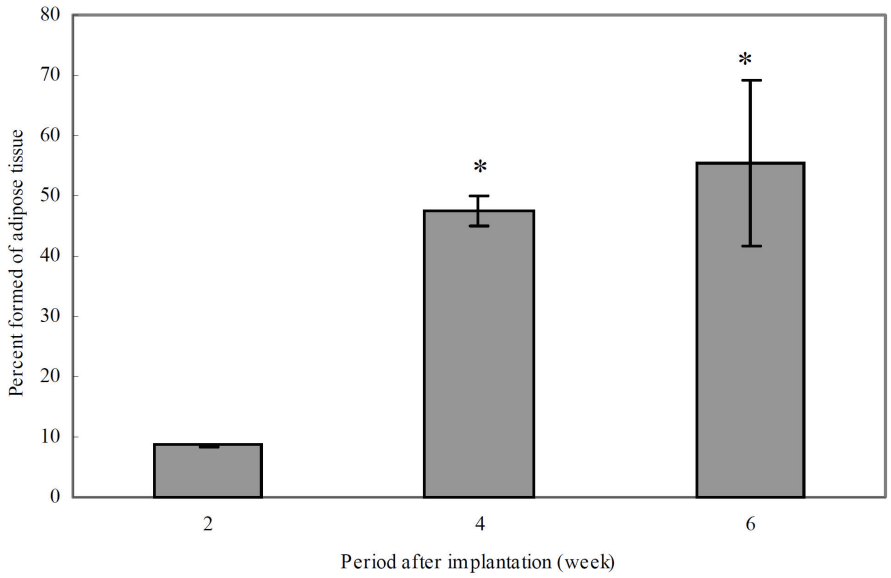

Figure 6. Time course of percentage of adipose tissue regenerated after implantation of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF. *, $p<0.05$; significant against the percentage of adipose tissue regenerated 2 weeks after implantation.

preadipocytes alone was much less effective, whereas no formation of adipose tissue was observed in the scaffold alone. Figure 8 shows the percent area of adipose tissue regenerated in the rat fat pad 4 weeks after implantation. The area of adipose tissue regenerated from the implantation of collagen- PP scaffold incorporating gelatin microspheres incorporating bFGF was larger compared with that of collagen- PP scaffold incorporating preadipocytes. Adipose tissue was not detected in the control group of collagen-PP scaffold without the controlled release of bFGF and preadipocytes.

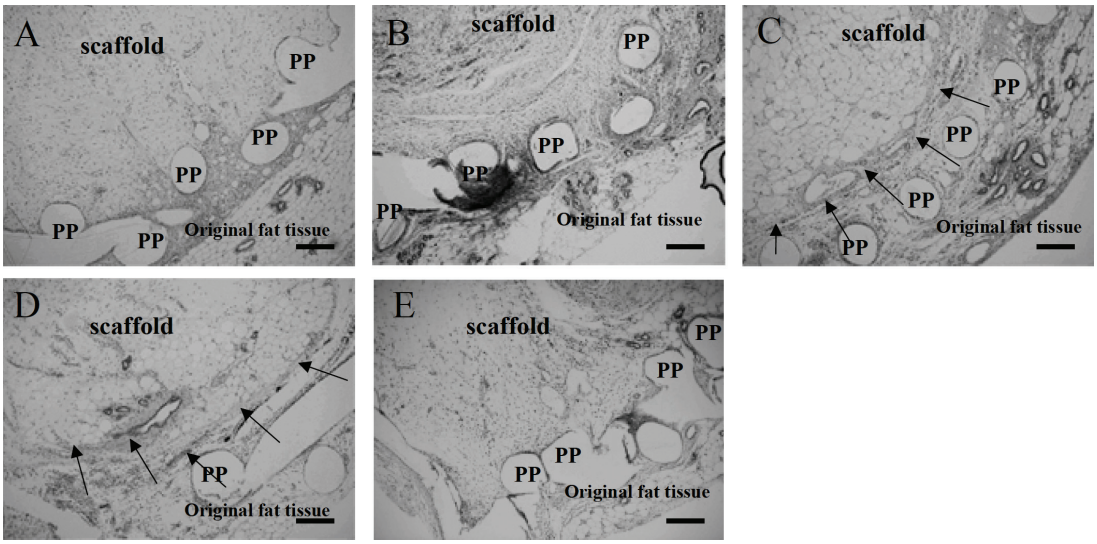

Figure 7. Histological sections of site implanted of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF and/or 1×10^5 preadipocytes 4 weeks post-operatively. Each section shows collagen-PP scaffold without bFGF and preadipocytes (A) (Group V (PBS)), collagen-PP scaffold with 1×10^5 preadipocytes (B) (Group III), collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF (C) (Group I (1.0)), collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF and 1×10^5 preadipocytes (D) (Group II), and collagen-PP scaffold mixed with 1.0 μg of free bFGF (E) (Group VI). (HE staining, Scale bar = 200 μm)

Influence of bFGF release profile on the *in situ* regeneration of adipose tissue

Figure 9 shows the histological sections of the site implanted 4 weeks after implantation of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF with different water contents. Many cells were observed in the HE histological section after implantation of collagen-PP scaffold incorporating gelatin microspheres containing bFGF with a water content of 90 %, but mature adipocytes accumulating lipids were not observed. On the other hand, mature adipocytes accumulating lipids were observed in the scaffold incorporating gelatin microspheres

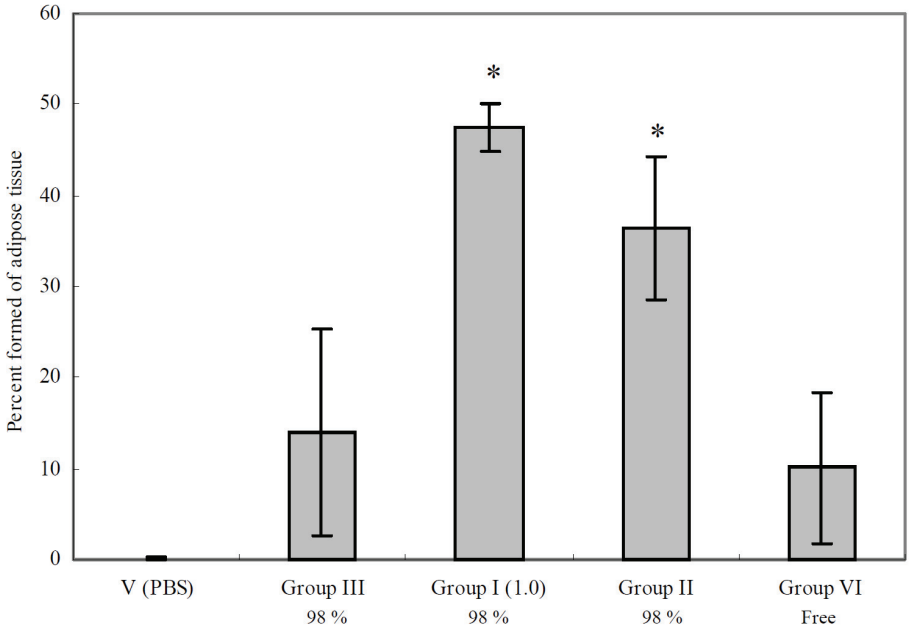

Figure 8. Effect of the rat syngeneic preadipocytes on the percentage of adipose tissue regenerated after implantation of collagen-PP scaffold with or without incorporation of gelatin microspheres containing 1.0 μg of bFGF. *, $p<0.05$; significant against the percentage of adipose tissue regenerated at the scaffold incorporating preadipocytes without gelatin microspheres containing 1.0 μg of bFGF (Group III).

containing 1 μg of bFGF with water contents of 95 and 98 %. Figure 10 shows dependence of the bFGF release profile on the *in situ* regeneration of adipose tissue. The regeneration was assessed by determining the area percentage of adipose tissue to the total area within the PP mesh on the histological sections 4 weeks after co-implantation of collagen scaffold and gelatin microspheres containing 1 μg of bFGF with different water contents (90, 95, 98, and 99 %). When gelatin microspheres with a water content of 95 or 98 % were used as the release carrier of bFGF, the amount of adipose tissue regenerated was significantly higher than that of gelatin microspheres

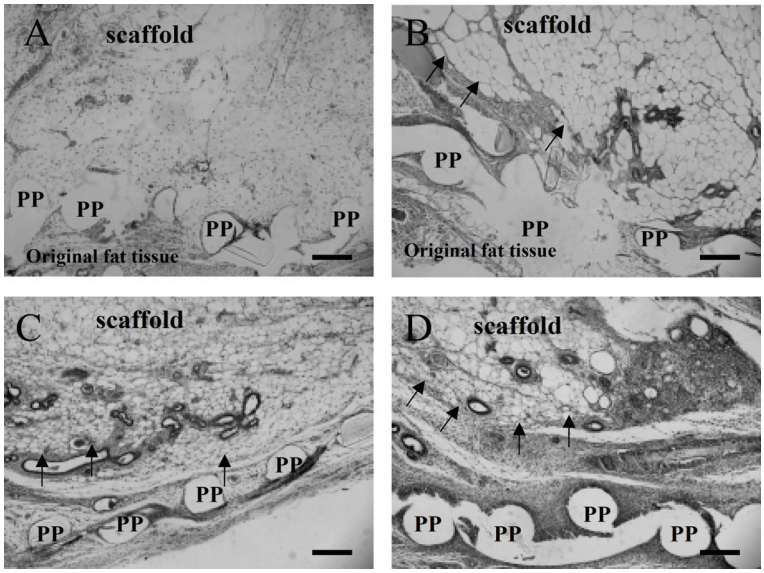

Figure 9. Histological sections of the site implanted of collagen-PP scaffold incorporating gelatin microspheres containing 1.0 μg of bFGF at water contents of 90 (A), 95 (B), 98 (C), and 99 % (D). (HE staining, Scale bar = 200 μm)

containing the same dose of bFGF with a water content of 90 %.

DISCUSSION

It has been designed an animal model to evaluate the regeneration of adipose tissue in the defect of fat pad and demonstrated five major findings for *in situ* regeneration of adipose tissue. These include the necessity of controlled release of bFGF for regeneration of adipose tissue, an optimal concentration of bFGF, an optimal time period of bFGF release, unnecessity of syngeneic preadipocytes, and feasible scaffold of type I collagen.

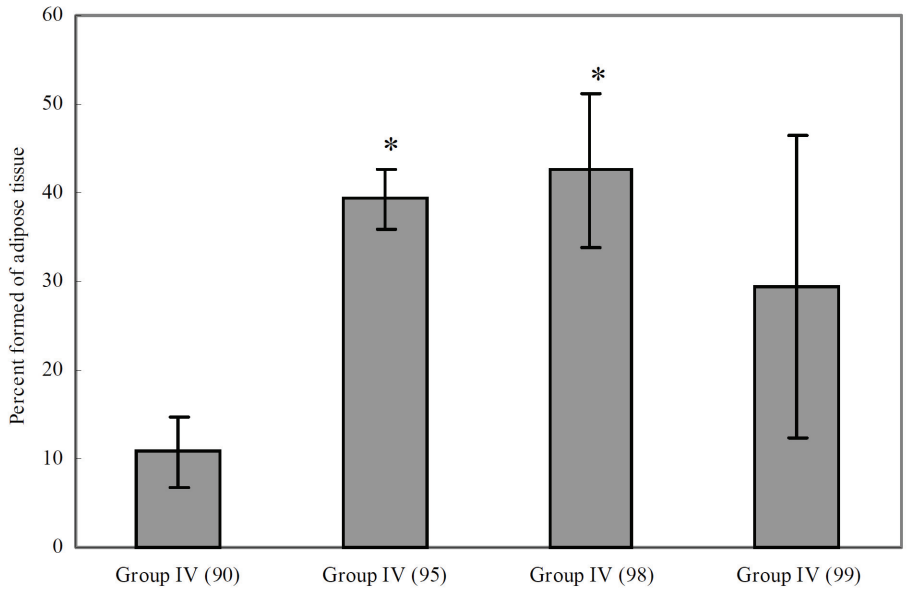

Figure 10. Effect of the water content of gelatin microspheres containing 1 μg of bFGF on the percentage of adipose tissue regenerated 4 weeks after implantation of collagen-PP scaffolds. *, $p<0.05$; significant against the percentage of adipose tissue regenerated at the scaffold containing gelatin microspheres incorporating 1.0 μg of bFGF at a water content of 90 % (Group IV (90)).

The defect model of fat pad was prepared to properly simulate the tissue environment of breast after partial mastectomy. That is, the defect is surrounded with natural adipose tissue. Some tissue-engineering trials have been reported on adipose tissue engineering in mouse or rat subcutis [21, 23, 29-33]. However, if adipose tissue engineering is applied to surgical reconstruction of a mammal defect, the defect surgically prepared is mostly surrounded by natural adipose tissue. In this chapter, orthotopic regeneration of adipose tissue is investigated to compare with that in the ectopic site. Controlled release of bFGF was a key technology for adipose tissue engineering. This is because the collagen sponge combined with free bFGF resulted in

poor adipogenesis compared with the scaffold combined with gelatin microspheres containing bFGF (Figures 7 and 8). The present study clearly indicated that an environment suitable to induce regeneration of adipose tissue could be created by combination of a collagen scaffold with the bFGF release system. There will be several reasons to be considered for the bFGF effect on regeneration of adipose tissue. It is possible that the controlled release of bFGF induced angiogenesis, resulting in efficient proliferation and maturation of adipose precursor cells migrated into the advance angiogenesis induced scaffold because of good oxygen and nutrients supply to the cells. Indeed, the previous studies about regeneration of adipose tissue with Matrigel indicated that angiogenesis induced by bFGF release could generate a good environment for tissue regeneration [32]. bFGF itself acts on the preadipocytes to accelerate their proliferation [34], or other growth factors that are provided by the bFGF-induced vasculature enables the cells to proliferate [35]. It is conceivable that the collagen scaffold plus the preadipocytes without the bFGF release system did not induce angiogenesis enough to maintain the survival of cells transplanted, resulting in no regeneration of adipose tissue. The collagen scaffold does not function as the carrier for bFGF release [36], which will cause poor angiogenesis in the collagen scaffold combined with preadipocytes and free bFGF. As a result, it is possible that the scaffold-cells-free bFGF combination results in poor regeneration of adipose tissue compared with the scaffold-cells-released bFGF. The direct effect of bFGF on preadipocytes is still controversial. It has been reported that bFGF has no adipogenic effect on human precursor cells [37] and shows an inhibitory effect on the regeneration of adipose tissue of rat preadipocytes [38]. On the other hand, sheep preadipocytes are

reported to differentiate in a culture medium containing bFGF [34]. In addition, it has been demonstrated that bFGF exhibits an accelerating effect on the adipose tissue regeneration of human precursor cells [39] or an enhancing adipogenic effect on rat precursor cells [40]. In the research studies, bFGF is applied in the solution form, which is different from the application form of bFGF in this study. Although the adipogenic effect of bFGF is not clear at the moment, it is conceivable that the controlled release of bFGF increases the number of preadipocytes and the rate of adipocyte differentiation, resulting in totally enhanced regeneration of adipose tissue. The adipogenic effect of bFGF should be considered. The bFGF dose dependence indicated that there was an optimal concentration range of bFGF for *in situ* regeneration of adipose tissue (Figures 3 and 4). It was reported that the amount of adipose tissue regenerated increased with an increase in the concentration of bFGF, which was immobilized in Matrigel [23]. On the other hand, there was an optimal dose of bFGF controlled release from gelatin microspheres for adipose tissue formation, and there was an optimal dose of bFGF immobilized in gelatin microspheres for capillary density at 2 weeks after implantation [29]. The optimal dose of bFGF from gelatin microspheres was the same in Chapter 1. Probably, a low dose of bFGF is not enough to exert its angiogenic or adipogenic effect even though the bioactive bFGF is released from the gelatin microspheres. On the other hand, when the bFGF dose is too high, in addition to the two effects of fibrous tissues into the collagen scaffold would become pronounced. A high dose of bFGF caused an inflammatory response at the implanted collagen scaffold. It may be that inflammation at the scaffold is too severe to induce tissue regeneration.

Cellularity in the collagen scaffold incorporating the controlled release of

bFGF and/or preadipocytes changed with time after implantation. There were many cells in the scaffold 2 weeks after implantation, but the majority were fibroblast-like cells and there were few matured adipocytes. The period of bFGF release from the gelatin microspheres with a water content of 98 % was about 2 weeks, whereas the increase in the percent of adipose tissue regenerated was observed between 2 and 4 weeks (Figures 5 and 6). This time lag may be explained based on the biological action of bFGF described above. It is possible that the bFGF release would enable preadipocytes to infiltrate into the scaffold and to increase their number. Thereafter, it needs a few weeks to infiltrate into the scaffold and subsequently differentiate the increased preadipocytes into matured fat cells *in vivo*. The percentage of adipose tissue regenerated was almost similar between 4 and 6 weeks of implantation. The maximum percent of adipose tissue regenerated was about 50 %. It was noted that the center area of the scaffold and the area close to the polypropylene mesh performed poorly. It was suggested that preadipocytes were infiltrated from the original adipose tissue by the controlled release of bFGF, but there were few adipocytes close to the PP mesh in some cases, and inflammatory cells were observed. We think that because some collagen-PP scaffolds had a small gap between the collagen sponge and PP mesh, preadipocytes could not attach to the collagen scaffold, and infiltrated focal inflammatory cells remained there. Although the histological site of adipose tissue regenerated seemed to be different from that of the original adipose tissue in terms of the size of matured adipocyte, it might be considered that it was still not enough blood supply in this size of scaffold. The ability of bFGF-incorporated microspheres to induce *in situ* regeneration of adipose tissue depended on their water content (Figures 9 and 10). It was possible

that the slow degrading gelatin microsphere contributed to prolonged retention of bFGF. When the rate of microsphere degradation was too slow compared with that of adipose tissue regeneration at the skull defect in the fat tissue, it was thought that induced rat preadipocytes derived from around original fat tissue were not fully differentiated to mature adipocytes. On the other hand, free bFGF and VI (99%) did not significantly enhance the percent area of adipose tissue regenerated at the rat skull model after 4 weeks implantation. This indicated that controlled release of bFGF was very important for *in situ* regeneration of adipose tissue. It was indicated that a balance in the time profile between the bFGF release and the *in situ* regeneration of adipose tissue was essential for the bFGF-induced regeneration of adipose tissue in the collagen-PP scaffold. This chapter clearly indicated that *in situ* regeneration of adipose tissue was not necessary to add preadipocytes (Figures 7 and 8). The Matrigel plus bFGF without preadipocytes induced adipogenesis in the subcutis in Chapter 4, whereas no adipogenesis by the combination of bFGF and a collagen I sponge was observed in the mouse subcutis in Chapter 1. In this chapter, adipogenesis by the combination of bFGF and a collagen I sponge was observed in the rat fat pad. The difference of adipogenesis can be explained in terms of different environment. It is conceivable that Matrigel® gives preadipocytes infiltrated a better environment for their differentiation. This is because it contained some growth factors and extracellular matrix (ECM) components suitable for de novo regeneration of adipose tissue. Preadipocytes were unnecessary in our study because many preadipocytes are present in the original adipose tissue. It is possible that preadipocytes were recruited into the scaffold by the controlled release of bFGF. It has been recognized in recent cell biology that preadipocytes are multipotent

for differentiation [41]. Preadipocytes are committed or determined to differentiate into fat cells, and the cell differentiation can be promoted depending on the microenvironment [19, 33]. It is well recognized that the number of adipocytes and their precursor cells are only less than half that of the total cells present in the adipose tissue and the remaining cells are vascular-related cells, such as various blood cells, endothelial cells, and pericytes [17]. After 4 weeks implantation of the collagen-PP scaffold incorporating gelatin microspheres containing 1 μg of bFGF and syngeneic rat preadipocytes into rat femoral muscle, not muscle tissue but adipose tissue was observed in the histological section (data not shown). This indicated that rat syngeneic preadipocytes differentiated easily into adipocytes. It was considered, however, that addition of preadipocytes was not affected well because there were many preadipocytes around the collagen-PP scaffold. The scaffold is one of the important factors for adipose tissue engineering. Implantable materials used for adipose tissue engineering have predominantly been porous biodegradable polymer foams [16, 42]. For instance, poly(L-lactic co-glycolic) acid scaffolds preseeded with preadipocytes have demonstrated regeneration of adipose tissue [30]. However, synthetic polymer foams will probably not be the optimal choice for many applications as they are too rigid and would be uncomfortable for patients. A more appropriate choice may be a biodegradable collagen sponge reinforced by poly(glycolic acid) fiber [43]. Nonbiodegradable scaffolds have also been investigated. For instance, Kral and Crandall recently demonstrated that the attachment and proliferation of preadipocytes on fluorotez monofilament-expanded polytetrafluoroethylene scaffolds coated with various extracellular matrices [44]. Matrigel® is an essential material to induce de novo

regeneration of adipose tissue [23, 32]. However, because the Matrigel® scaffold is a mouse tumor-derived material, it is practically impossible to apply it to humans. Moreover, in the case of gel-type scaffold, the degradation of gel is necessary to allow cells to infiltrate into the scaffold because the pore size of gel is not large enough for cell infiltration [45]. On the other hand, for the sponge-type scaffold, it is likely that cells readily infiltrates into the scaffold *in vivo* without degradation of scaffold [22]. The optimal scaffold for adipose tissue engineering remains unclear. Modification with adhesive bioactive substances will give the scaffold new biological functions. However, this strategy is complicated by the fact that the constitution and distribution of the ECMs further varies adipocyte differentiation [46]. Although an optimal matrix for preadipocyte is not yet known, type I collagen is a material widely used in tissue regeneration and a porous collagen matrix supports cellular ingrowth and new matrix synthesis. Indeed, preadipocytes readily adhere to laminin-1 as compared to other ECMs, but preadipocytes also adhere to type I collagen [47]. Chapter 3 revealed that the coating of mixed basement membrane components and type I collagen promoted proliferation and adipogenic differentiation of human preadipocytes. In this reconstructed model of adipose tissue, we used a type I collagen scaffold. When large enough numbers of original preadipocytes exist, a scaffold of type I collagen incorporating controlled release of bFGF has induced infiltration of cells from original fat tissue, their growth, and adipogenesis.

REFERENCES

1. Carlson GW. Breast Reconstruction. Surgical Options and Patient Selection. Cancer 1994;74:436-9.
2. Chong GC, Masson JK, Woods JE. Breast Restoration after Mastectomy for Cancer. Mayo Clin Proc 1975;50:453-8.
3. Hartrampf CR, Scheflan M, Black PW. Breast Reconstruction with a Transverse Abdominal Island Flap. Plast Reconstr Surg 1982;69:216-25.
4. Huss FR, Kratz G. Mammary Epithelial Cell and Adipocyte Co-Culture in a 3-D Matrix: The First Step Towards Tissue-Engineered Human Breast Tissue. Cells Tissues Organs 2001;169:361-7.
5. Rosen PB, Hugo NE. Augmentation Mammaplasty by Cadaver Fat Allografts. Plast Reconstr Surg 1988;82:525-6.
6. Billings E, Jr., May JW, Jr. Historical Review and Present Status of Free Fat Graft Autotransplantation in Plastic and Reconstructive Surgery. Plast Reconstr Surg 1989;83:368-81.
7. Ellenbogen R. Free Autogenous Pearl Fat Grafts in the Face--a Preliminary Report of a Rediscovered Technique. Ann Plast Surg 1986;16:179-94.
8. Ersek RA. Transplantation of Purified Autologous Fat: A 3-Year Follow-up Is Disappointing. Plast Reconstr Surg 1991;87:219-27; discussion 28.
9. Fagrell D, Enestrom S, Berggren A, Kniola B. Fat Cylinder Transplantation: An Experimental Comparative Study of Three Different Kinds of Fat Transplants. Plast Reconstr Surg 1996;98:90-6; discussion 7-8.

10. Peer LA. The Neglected Free Fat Graft. Plast Reconstr Surg 1956;18:233-50.
11. Smahel J. Failure of Adipose Tissue to Heal in the Capsule Preformed by a Silicone Implant. Eur J Plast Surg 1985;8:109-15.
12. Smahel J. Experimental Implantation of Adipose Tissue Fragments. Br J Plast Surg 1989;42:207-11.
13. Tabata Y. The Importance of Drug Delivery Systems in Tissue Engineering. Pharmaceutical Science and Technology Today 2000;3:80-9.
14. Brey EM, Patrick CW, Jr. Tissue Engineering Applied to Reconstructive Surgery. IEEE Eng Med Biol Mag 2000;19:122-5.
15. Patrick CW, Jr. Adipose Tissue Engineering: The Future of Breast and Soft Tissue Reconstruction Following Tumor Resection. Semin Surg Oncol 2000;19:302-11.
16. Patrick CW, Jr., Mikos AG, McIntire LV. Frontiers in Tissue Engineering. New York: Pergamon, 1998.
17. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage Cells from Human Adipose Tissue: Implications for Cell-Based Therapies. Tissue Eng 2001;7:211-28.
18. Ailhaud G, Grimaldi P, Negrel R. Cellular and Molecular Aspects of Adipose Tissue Development. Annu Rev Nutr 1992;12:207-33.
19. Green H, Kehinde O. Formation of Normally Differentiated Subcutaneous Fat Pads by an Established Preadipose Cell Line. J Cell Physiol 1979;101:169-71.
20. De Ugarte DA, Ashjian PH, Elbarbary A, Hedrick MH. Future of Fat as Raw Material for Tissue Regeneration. Ann Plast Surg 2003;50:215-9.
21. von Heimburg D, Zachariah S, Heschel I, Kuhling H, Schoof H, Hafemann B,

Pallua N. Human Preadipocytes Seeded on Freeze-Dried Collagen Scaffolds Investigated in Vitro and in Vivo. Biomaterials 2001;22:429-38.

22. von Heimburg D, Zachariah S, Low A, Pallua N. Influence of Different Biodegradable Carriers on the in Vivo Behavior of Human Adipose Precursor Cells. Plast Reconstr Surg 2001;108:411-20; discussion 21-2.
23. Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De Novo Adipogenesis in Mice at the Site of Injection of Basement Membrane and Basic Fibroblast Growth Factor. Proc Natl Acad Sci U S A 1998;95:1062-6.
24. Ikada Y, Tabata Y. Protein Release from Gelatin Matrices. Adv Drug Deliv Rev 1998;31:287-301.
25. Tabata Y, Hijikata S, Ikada Y. Enhanced Vascularization and Tissue Granulation by Basic Fibroblast Growth Factor Impregnated in Gelatin Hydrogels. J Control Release 1994;31:189-99.
26. Tabata Y, Hijikata S, Muniruzzaman M, Ikada Y. Neovascularization Effect of Biodegradable Gelatin Microspheres Incorporating Basic Fibroblast Growth Factor. J Biomater Sci Polym Ed 1999;10:79-94.
27. Tabata Y, Ikada Y. Potentiated in Vivo Biological Activity of Basic Fibroblast Growth Factor by Incorporation Intopolymer Hydrogel Microsphere. 4th Japan International SAMPE Symposium 1995;4:577-82.
28. Tabata Y, Nagano A, Ikada Y. Biodegradation of Hydrogel Carrier Incorporating Fibroblast Growth Factor. Tissue Eng 1999;5:127-38.
29. Masuda T, Furue M, Matsuda T. Photocured, Styrenated Gelatin-Based Microspheres for De Novo Adipogenesis through Corelease of Basic Fibroblast

Growth Factor, Insulin, and Insulin-Like Growth Factor I. Tissue Eng 2004;10:523-35.

30. Patrick CW, Jr., Chauvin PB, Hobley J, Reece GP. Preadipocyte Seeded PLGA Scaffolds for Adipose Tissue Engineering. Tissue Eng 1999;5:139-51.
31. Patrick CW, Jr., Zheng B, Johnston C, Reece GP. Long-Term Implantation of Preadipocyte-Seeded PLGA Scaffolds. Tissue Eng 2002;8:283-93.
32. Tabata Y, Miyao M, Inamoto T, Ishii T, Hirano Y, Yamaoki Y, Ikada Y. De Novo Formation of Adipose Tissue by Controlled Release of Basic Fibroblast Growth Factor. Tissue Eng 2000;6:279-89.
33. Yuksel E, Weinfeld AB, Cleek R, Waugh JM, Jensen J, Boutros S, Shenaq SM, Spira M. De Novo Adipose Tissue Generation through Long-Term, Local Delivery of Insulin and Insulin-Like Growth Factor-1 by PLGA/PEG Microspheres in an in Vivo Rat Model: A Novel Concept and Capability. Plast Reconstr Surg 2000;105:1721-9.
34. Broad TE, Ham RG. Growth and Adipose Differentiation of Sheep Preadipocyte Fibroblasts in Serum-Free Medium. Eur J Biochem 1983;135:33-9.
35. Fukumura D, Ushiyama A, Duda DG, Xu L, Tam J, Krishna V, Chatterjee K, Garkavtsev I, Jain RK. Paracrine Regulation of Angiogenesis and Adipocyte Differentiation During in Vivo Adipogenesis. Circ Res 2003;93:e88-97.
36. Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In Vitro Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials 1998;19:1781-9.
37. Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, Takagishi K, Kato

Y. Retention of Multilineage Differentiation Potential of Mesenchymal Cells During Proliferation in Response to Fgf. Biochem Biophys Res Commun 2001;288:413-9.

38. Roncari DA, Le Blanc PE. Inhibition of Rat Perirenal Preadipocyte Differentiation. Biochem Cell Biol 1990;68:238-42.
39. Locklin RM, Oreffo RO, Triffitt JT. Effects of TGFbeta and bFGF on the Differentiation of Human Bone Marrow Stromal Fibroblasts. Cell Biol Int 1999;23:185-94.
40. Neubauer M, Fischbach C, Bauer-Kreisel P, Lieb E, Hacker M, Tessmar J, Schulz MB, Goepferich A, Blunk T. Basic Fibroblast Growth Factor Enhances PPARgamma Ligand-Induced Adipogenesis of Mesenchymal Stem Cells. FEBS Lett 2004;577:277-83.
41. Caplan AI. Mesenchymal Stem Cells. J Orthop Res 1991;9:641-50.
42. Lee KY, Halberstadt CR, Holder WD, Mooney DJ. Breast Reconstruction. In: Lanza RP, Langer R, Vacanti J, editors. Principles of Tissue Engineering, Second Edition. New York: Academic Press, 2000. p. 409-23.
43. Hiraoka Y, Kimura Y, Ueda H, Tabata Y. Fabrication and Biocompatibility of Collagen Sponge Reinforced with Poly(Glycolic Acid) Fiber. Tissue Eng 2003;9:1101-12.
44. Kral JG, Crandall DL. Development of a Human Adipocyte Synthetic Polymer Scaffold. Plast Reconstr Surg 1999;104:1732-8.
45. Yamamoto M, Tabata Y, Kawasaki H, Ikada Y. Promotion of Fibrovascular Tissue Ingrowth into Porous Sponges by Basic Fibroblast Growth Factor. Journal of materials science 2000;11:213-8.

46. Kubo Y, Kaidzu S, Nakajima I, Takenouchi K, Nakamura F. Organization of Extracellular Matrix Components During Differentiation of Adipocytes in Long-Term Culture. In Vitro Cell Dev Biol Anim 2000;36:38-44.
47. Patrick CW, Jr., Wu X. Integrin-Mediated Preadipocyte Adhesion and Migration on Laminin-1. Ann Biomed Eng 2003;31:505-14.

Chapter 6

Regeneration of capillary vessels by gelatin hydrogels containing stromal cell-derived factor (SDF)-1

INTRODUCTION

As a new strategy to treat defective tissues, regenerative medicine by tissue engineering has been expected. In tissue engineering, cells and the local environment composed of scaffolds and bioactive molecules, are combined for the induction of tissue regeneration [1]. It has been explored that biodegradable gelatin hydrogels designed for the controlled release of bioactive molecules, such as growth factors and plasmid DNAs, enhance their *in vivo* biological activities [2]. This release system is one of the drug delivery system (DDS) technologies to prolong their *in vivo* half-life period and consequently enhance the therapeutic efficacy at reduced injection doses. Some clinical trials of angiogenesis therapy by the release technology of basic fibroblast growth factor (bFGF) have been started in Japan to demonstrate the therapeutic feasibility [3].

A chemokine, stromal cell derived growth factor (SDF-1, CXCL12) is known as a chemokine which functions in the inflammation reaction, leukocyte development, and the maintenance and recruitment of stem cells [4-7]. Additionally, SDF-1 has an inherent ability to mobilize hematopoietic stem cells with the receptor CXCR4 from the

bone marrow [8].

In this chapter, the release technology of hydrogel was applied to SDF-1 and the biological potential was evaluated in terms of SDF-1-induced angiogenesis. Hydrogels were prepared from various gelatin derivatives with different physicochemical properties to achieve the controlled release of SDF-1. Following the subcutaneous implantation of gelatin hydrogels containing SDF-1 into the back of mice, angiogenesis was evaluated and compared with the results obtained by simple injection of SDF-1 solution. The *in vivo* profiles of SDF-1 release from gelatin hydrogels with different biodegradabilities were examined.

EXPERIMENTAL

Materials

Gelatin samples, prepared by an alkaline treatment of bovine bone collagen (the isoelectric point (IEP) = 5.0) and an acidic treatment of pig skin collagen (IEP=9.0), termed as the acidic type and basic type here, respectively, were kindly supplied by Nitta Gelatin Inc. (Osaka, Japan). Recombinant human stromal cell-derived factor-1 (SDF-1, 350-NS/CF) was obtained from R&D systems, Inc. (Minneapolis, MN). $Na^{125}I$ (NEZ-033H, >12.95 GBq/ml) and N'-succinimidyl-3-(4-hydroxy-3,5-di[^{125}I]iodo-phenyl)propionate ([^{125}I] Bolton-Hunter reagent, NEX-120H, 147 MBq/ml) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Glutaraldehyde (GA), glycine, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd.

(Osaka, Japan) and used without further purification.

Preparation of gelatin derivatives with various isoelectric points

Gelatin was chemically derivatized with succinic anhydride (Succ), decylamine (C10), spermine (SM), and ethylenediamine (ED) with or without 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as a catalyst to change the electronic nature of gelatin [9]. The IEP of derivatives was measured according to the method previously reported [10]. Briefly, 1 wt% of gelatin derivative solution was applied for an ion exchange column packed with mixed cationic (DOWEX 50W-X8) and anionic (DOWEX 1-X8) exchange resins at 40 °C and the pH of flow through was measured with a pH meter (D-22, HORIBA, Kyoto, Japan) at 40 °C.

Preparation of gelatin hydrogels

Aqueous solution of 5 wt% each gelatin derivative was mixed with various amounts of GA, followed by leaving at 4 °C for 12 hr for crosslinking gelatin. The gelatin hydrogel crosslinked was treated by 0.1 M glycine solution to block the residual aldehyde groups. After washing three times with double-distilled water (DDW), the hydrogels were freeze-dried. The crosslinking extent of hydrogels prepared was evaluated by measuring the water content according to the method previously described [11].

***In vitro* release test of SDF-1 from gelatin hydrogels**

SDF-1 was radioiodinated through the conventional chloramine T method [12].

$Na^{125}I$ (5 μl) was added to 200 μl of SDF-1 solution in 0.5 M potassium phosphate-buffer (pH 7.5) containing 0.5 M sodium chloride. Then, 0.2 mg/ml of chloramine-T in same buffer (100 μl) was added to the solution mixture. After agitation at room temperature for 2 min, 100 μl of phosphate-buffered saline solution (PBS, pH 7.5) containing 0.4 mg of sodium metabisulfate was added to the reaction solution to stop the radioiodination. The reaction mixture was passed through PD-10 desalting column (GE Healthcare Life Sciences, Giles, U.K.) to remove the uncoupled, free ^{125}I molecules from the ^{125}I-labeled SDF-1. PBS solution of ^{125}I-labeled SDF-1 (20 μl) was dropped onto the freeze-dried hydrogel of gelatin derivatives (2 mg, 2.5 x 2.5 x 3 mm^3), followed by leaving at 25 °C for 3 hr to obtain hydrogels containing ^{125}I-labeled SDF-1. For the *in vitro* release test, one hydrogel containing ^{125}I-labeled SDF-1 was agitated at 37 °C in 1 ml of PBS. The supernatant was removed at 0.5, 1, 2, 4, 8, and 24 hr later and replaced with the same volume of fresh PBS. The radioactivity of each supernatant was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan) to evaluate the time profile of SDF-1 release (n=3, at each time point).

In vivo release test of SDF-1 from gelatin hydrogels

All the surgical and observation procedures were performed under continuous inhalation anesthesia by isoflurane (Forane®, Abbott Japan Ltd., Osaka, Japan) with 400 anesthesia unit (Univentor Ltd., Zejtun, Malta). PBS containing ^{125}I-labeled SDF-1 (20 μl) was dropped onto 2 mg of freeze-dried gelatin hydrogels, followed by incubation at 25 °C for 3 hr to allow to swell into the hydrogel. Following the implantation of gelatin hydrogels containing ^{125}I-labeled SDF-1 into the back subcutis of female ddY mice, 6

week-age (18-20 g body weight, Shimizu Laboratory Supply, Kyoto, Japan), tissue around the site implanted was extracted at different time intervals after hydrogel implantation, and the tissue radioactivity was counted by the gamma counter to estimate the *in vivo* time profiles of SDF-1 release (n=3, at each time point).

In vivo evaluation of gelatin hydrogel degradation

To evaluate degradation profiles of gelatin hydrogels, the *in vivo* implantation of ^{125}I-labeled hydrogels was performed [12, 13]. [^{125}I] Bolton-Hunter reagent solution (20 μl) in benzene was completely evaporated under dry nitrogen. The resultant reagent was dissolved into 1 ml of PBS (pH 7.5). The reagent solution was impregnated into the gelatin hydrogel sheet (2.5 x 2.5 x 3 mm^3) at a volume of 20 μl per sheet. The sheets were incubated at 4 °C for 3 hr to introduce ^{125}I into the amino groups of gelatin. The radioiodinated sheets were washed with DDW thoroughly (4 °C, 4 days) to exclude non-reacted ^{125}I reagent, till the radioactivity of DDW returned to a background level. ^{125}I-labeled gelatin hydrogels were implanted into the back subcutis of mice. Then, tissue around the sites implanted was extracted at different time intervals after hydrogel implantation, and the tissue radioactivity was counted by the gamma counter to obtain the degradation profiles of hydrogels over time (n=3, at each time point).

RT-PCR

After 3-day implantation of gelatin hydrogels containing SDF-1 into back subcutis, 15 x 15 mm^2 of skin tissues around the site implanted was taken out by a surgical scalpel. The total RNA was extracted by using RNeasy fibrous tissue mini kit

(Qiagen Inc., Valencia, VA) according to the manufacturer's instructions. Reverse transcription reaction was performed with the SuperScript II First-Stand Synthesis System (Invitrogen Co., Carlsbad, NM). Real time PCR was performed on Prism 7500 real time PCR thermal cycler (Applied Biosystems Inc., Foster City, CA) from 10 ng of cDNA in a total volume of 25 μl containing POWER SYBR Green PCR Master Mix (Applied Biosystems) and 10 μM of each primer (Table 1). The reaction mixture was incubated for the initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles. Each cycle consisted of the following three steps; 94 °C for 15 sec, 57 °C for 15 sec, and 72 °C for 1 min. Each messenger RNA (mRNA) level was normalized by the expression level of 18S ribosomal RNA as an internal control.

Regeneration assay of capillary vessels by gelatin hydrogels containing SDF-1

The biological activity of SDF-1 incorporated in gelatin hydrogels was evaluated by using a skinfold chamber attached on the back skin of mice, according to the study from Ichioka *et al.* [14]. The gelatin hydrogel containing 5 μg of SDF-1 was implanted into the skin defect in the chamber 3 days after the chamber attachment. The number of capillaries regenerated around the hydrogel implanted was microscopically counted at different time intervals using Stemi 2000C stereomicroscope (Carl Zeiss Japan, Tokyo, Japan).

Table 1. Primer sequences used for real time PCR

Name	Primer	
CXCR4	sense	GCTGGCTGAAAAGGCAGTCTAT
	antisense	TGACGTCGGCAAAGATGAAGT
18S	sense	ACTCAACACGGGAAACCTCA
	antisense	AACCAGACAAATCGCTCCAC

Statistical analysis

All the results were expressed as the mean ± standard deviation. For statistical analysis, the Tukey-Kramer post-test for multiple comparisons was used and differences were considered to be significant at $p < 0.05$.

RESULTS

Characterization of gelatin derivatives and their hydrogels

Table 2 shows the isoelectric point and degree of substitution of gelatin derivatives prepared, and the water content of their hydrogels crosslinked with GA. pI5 and pI9 gelatin were alkaline and acid-processed gelatin raw materials. Thus, the degree of substitution was not indicated. Succinylation of gelatin decreased the IEP while the derivatization with spermine and ethylenediamine increased it. Chemical

Table 2. Isoelectric points and degree of substitution of gelatin derivatives used for hydrogel preparation, and water contents of the hydrogels crosslinked with GA; succinylated gelatin (Succ), decylamine-introduced gelatin (C10), acidic type gelatin (PI5), basic type gelatin (pI9), spermine-introduced gelatin (SM), and ethylenediamine- introduced gelatin (ED)

	Succ	C10	pI5	pI9	SM	ED
IEP	4.57	4.72	5.09	8.90	10.83	11.21
Degree of substitution (mol/mol %)	29.0 ± 1.2	28.6 ± 2.4	-	-	49.0 ± 1.1	50.9 ± 1.1
Water content of hydrogels(wt%)	97.9 ± 0.0	97.4 ± 0.2	97.8 ± 0.2	98.2 ± 0.1	97.4 ± 0.8	97.9 ± 0.6

derivatization modified the electric charge of gelatin. Table 3 shows the water content of hydrogels prepared from succinylated gelatin. The water content was changed by altering the GA concentration in hydrogel preparation. For *in vitro* and *in vivo* release experiment, hydrogels from gelatin derivatives with water contents of 97-98% were used.

Figure 1 shows the time profile of SDF-1 release from various gelatin hydrogels. The release profiles depended on the type of gelatin hydrogels. Irrespective of the hydrogel type, the amount of SDF-1 released from the gelatin hydrogels reached to a certain level and saturated. Higher suppression of *in vitro* SDF-1 release was observed for the hydrogel with Succ. Figure 2 shows the effect of hydrogel type on the SDF-1 release. The highest amount of SDF-1 remaining was observed for the Succ-derivatized hydrogel.

Table 3. Preparation conditions and water content of succinylated gelatin hydrogels

Gelatin concentration (wt%)	Glutaraldehyde concentration (mM)	Water content (wt%)
5	44.9	95.8 ± 0.1
5	22.4	96.3 ± 0.1
5	11.2	97.9 ± 0.0
5	6.24	98.8 ± 0.1

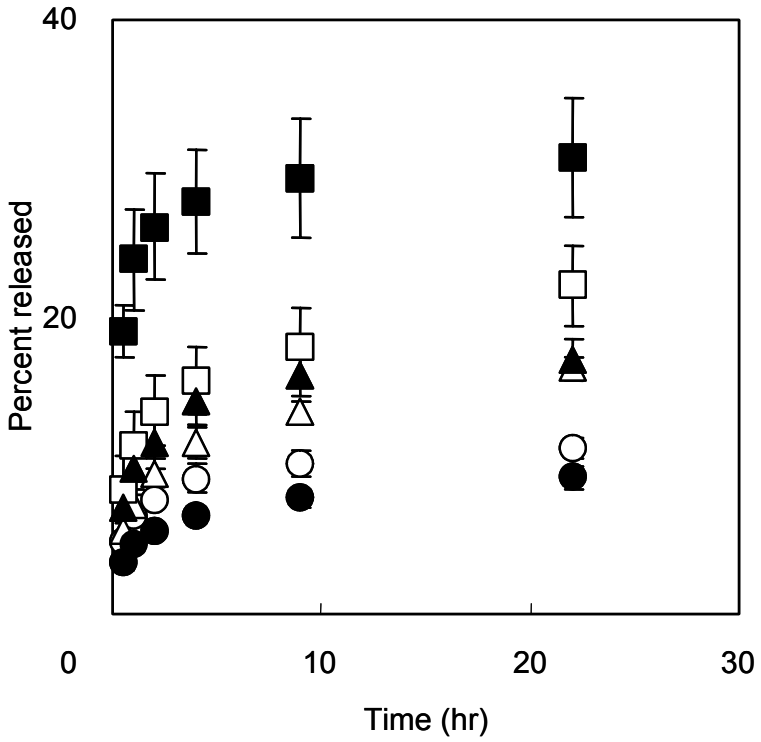

Figure 1. *In vitro* release profiles of SDF-1 from hydrogels of gelatin-derivatives; succinylated gelatin (Succ) (○), decylamine-introduced gelatin (C10) (△), acidic type gelatin (PI5) (□), basic type gelatin (pI9) (●), spermine-introduced gelatin (SM) (▲), and ethylenediamine-introduced gelatin (ED) (■).

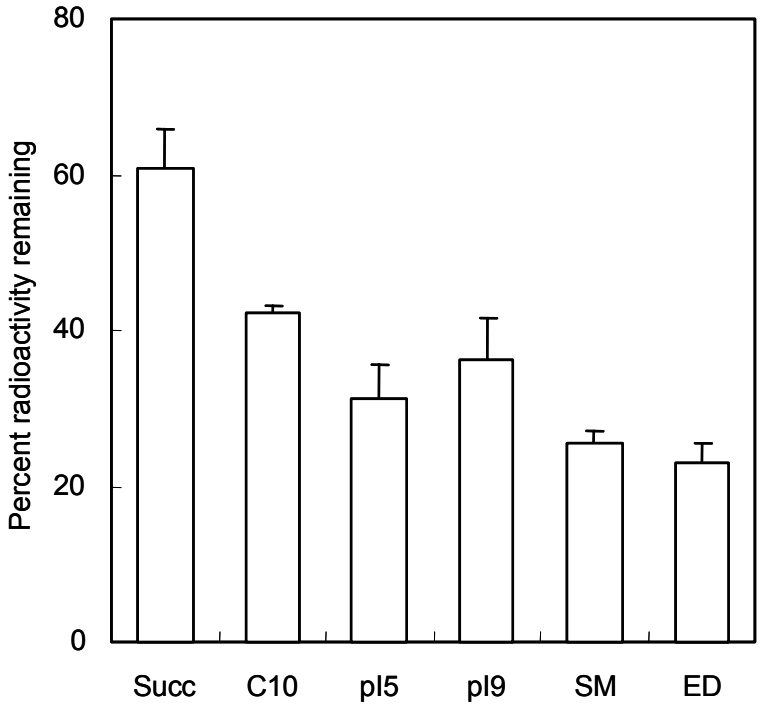

Figure 2. Percent radioactivity remaining of gelatin hydrogels containing ^{125}I-labeled SDF-1 24 hr after subcutaneous implantation. Abbreviations on the X-axis are indicated in Figure 1.

***In vivo* profiles of SDF-1 release and gelatin hydrogels degradation**

Figure 3 shows the time profiles of *in vivo* radioactivity remaining after implantation of Succ gelatin hydrogels containing ^{125}I-labeled SDF-1 with different water contents. For the hydrogel containing ^{125}I-labeled SDF-1, the radioactivity was retained for longer time periods than the solution of ^{125}I-labeled SDF-1. The radioactivity of every hydrogel decreased with the implantation time. The gelatin hydrogels with higher water contents released SDF-1 faster than those with lower water contents. Figure 4 shows the time profiles of *in vivo* radioactivity remaining after implantation of ^{125}I-labeled gelatin hydrogels with different water contents. Similarly to

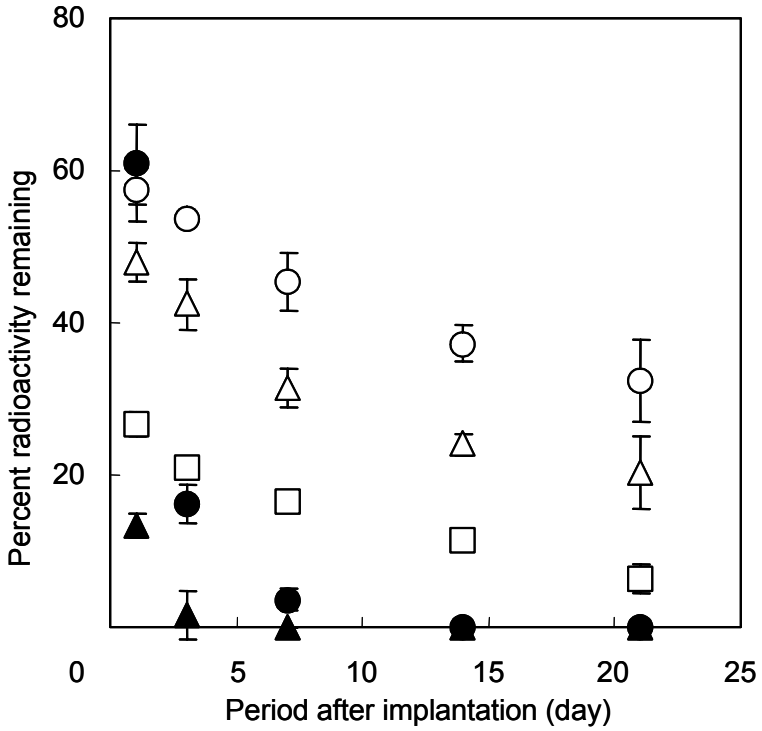

Figure 3. Time profiles of radioactivity remaining after subcutaneous implantation of gelatin hydrogels containing ^{125}I-labeled SDF-1 with different water contents of 95.8 (○), 96.3 (△), 97.9 (□), and 98.8 wt% (●) or subcutaneous injection of ^{125}I-labeled SDF-1 solution (▲).

Figure 3, the gelatin hydrogels with higher water contents were degraded faster than those with lower water contents. Figure 5 shows the correlation of radioactivity remaining after implantation between the gelatin hydrogels containing ^{125}I-labeled SDF-1 and ^{125}I-labeled gelatin hydrogels. The time profile of SDF-1 remaining was in good accordance with that of hydrogel remaining.

Regeneration of capillary vessels by gelatin hydrogels containing SDF-1

Figure 6 shows the mRNA expression of CXCR4, the receptor of SDF-1, around the site implanted with gelatin hydrogels containing various amounts of SDF-1.

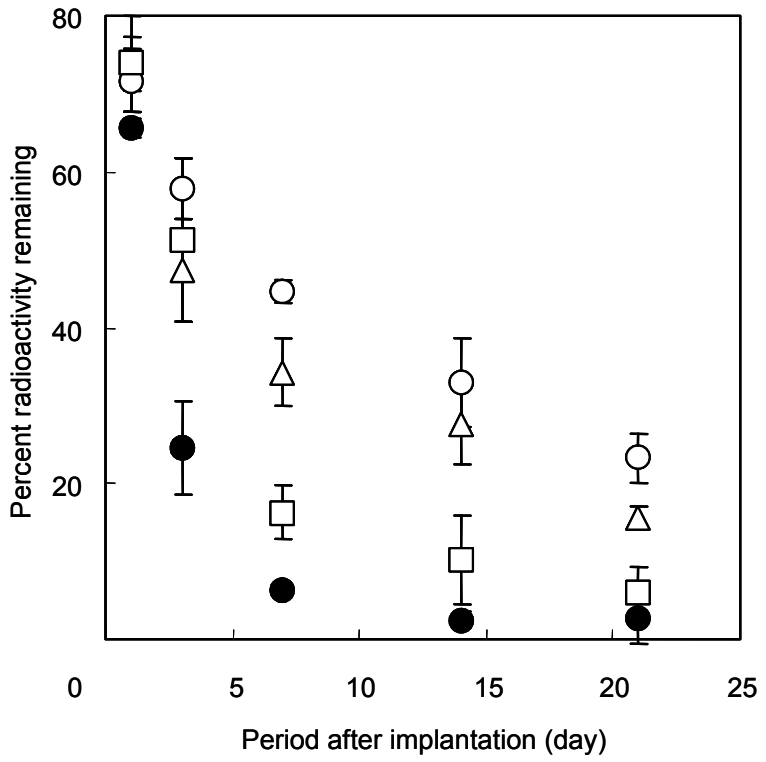

Figure 4. Time profiles of radioactivity remaining after subcutaneous implantation of ^{125}I-labeled gelatin hydrogels with different water contents of 95.8 (○), 96.3 (△), 97.9 (□), and 98.8 wt% (●).

The expression level was up-regulated with the dose of SDF-1 implanted. The level was significantly high at the dose of 5 μg/site compared with that of other doses.

Figure 7 shows the light microscopic photographs of tissue site 3 days after implantation of gelatin hydrogel containing SDF-1. Figure 8 shows the time profiles of the number of blood vessel capillaries regenerated in the tissue after implantation of gelatin hydrogels containing SDF-1. Significantly larger number of capillaries was observed at the site implanted with the hydrogel containing SDF-1 4 and 7 days after implantation than the case of the SDF-1-injected group. However, no significance was

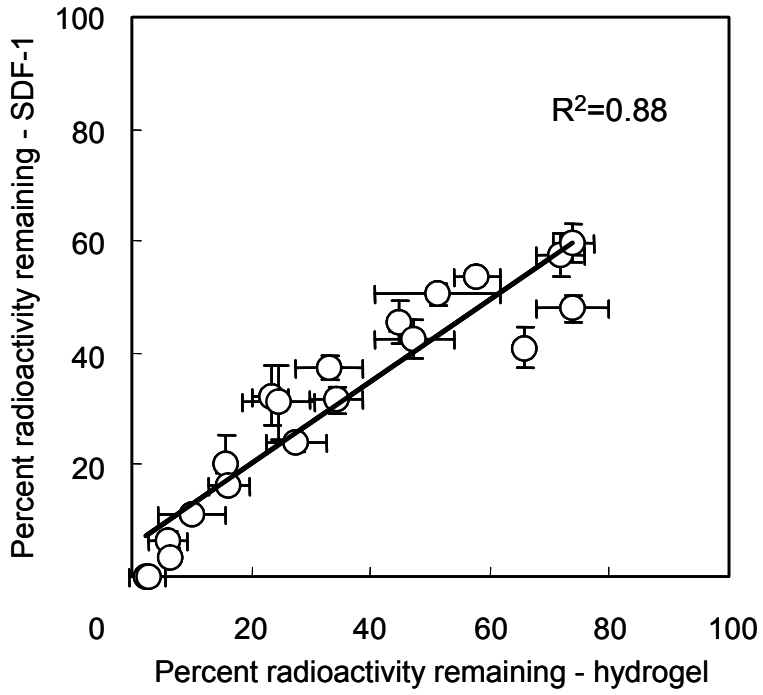

Figure 5. Relationship of remaining radioactivity after subcutaneous implantation into the mouse back between gelatin hydrogels containing ^{125}I-labeled SDF-1 and ^{125}I-labeled hydrogels of release carrier.

observed in the capillary number among all the experimental groups 10 days after treatment.

DISCUSSION

This study clearly demonstrates that the controlled release of SDF-1 could be achieved by gelatin hydrogels. The SDF-1 release was experimentally confirmed to be effective in enhancing SDF-1-induced angiogenesis. The Succ hydrogel was suitable for

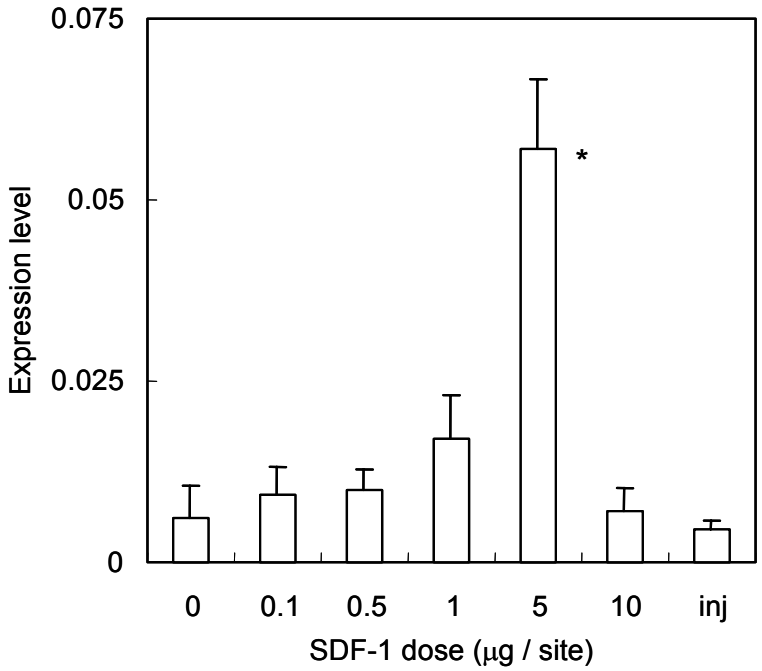

Figure 6. CXCR4 mRNA expression at the site implanted of gelatin hydrogels (water content: 98.8 wt%) containing various amounts of SDF-1 3 days after implantation. As control, mRNA expression was examined after injection of SDF-1 solution (5 μg) (inj). *: $p<0.05$, significant against the expression level of other groups.

SDF-1 release. The SDF-1 release enhanced the *in vivo* regeneration of capillary vessels to a significantly great extent compared with the SDF-1 solution.

SDF-1 bears positive charge in the physiological pH condition (pH 7.4) because of its IEP of 10.26. It is possible that SDF-1 molecules can electrostatically interact with the succinylated gelatin of negative charge, resulting in the suppressed SDF-1 release from the succinylated hydrogel accompanied with biodegradation. This hydrogel degradation-based SDF-1 release is experimentally confirmed by the *in vitro* and *in vivo* tests of SDF-1 release (Figures 1 and 2). Irrespective of the hydrogel type,

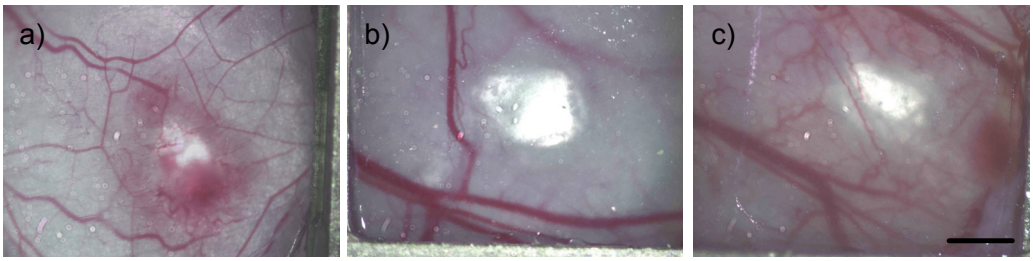

Figure 7. Light microscopic photographs of tissue site 3 days after implantation of gelatin hydrogel (water content: 98.8 wt%) containing 5 μg of SDF-1 (a) and empty gelatin hydrogel (b) or injection of SDF-1 solution (5 μg) (c). Scale bar = 1 mm.

the amount of SDF-1 released *in vitro* from gelatin hydrogels increased with time to attain a certain level and thereafter saturated. In this release system, the SDF-1 molecules are immobilized in the hydrogel through their physicochemical interaction with the gelatin networks. Without the enzymatic degradation of hydrogels to generate water-soluble gelatin fragments, SDF-1 molecules immobilized are not released from the hydrogels. Based on this release mechanism, all the SDF-1 molecules are not released under the *in vitro* conditions in the absence of enzymes where the hydrogels is not degraded. The initial release of SDF-1 is due to the simple diffusion of free SDF-1 non-immobilized in the hydrogel. This release profile was identical with that of other growth factors reported previously [9, 15]. The SDF-1-gelatin interaction would suppress the SDF-1 release from the hydrogel under the *in vitro* condition where the hydrogel is not degraded. Such a suppressed effect, though low, was observed for other types of hydrogels under the *in vitro* condition where the hydrogel is not degraded. This can be explained by different interaction forces between SDF-1 and gelatin molecules.

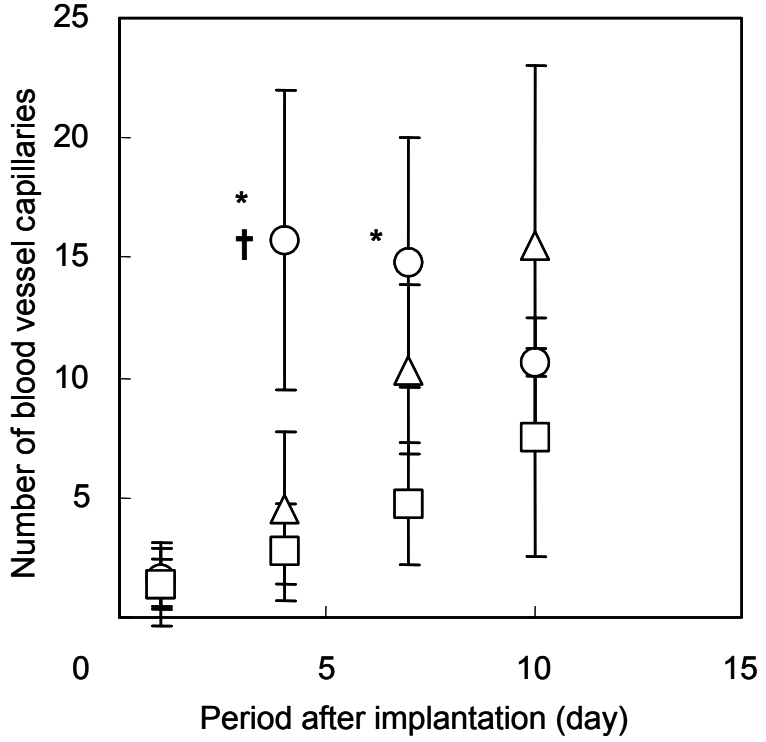

Figure 8. The number of blood vessel capillaries regenerated at the tissue site at different time intervals after implantation of gelatin hydrogels (water content: 98.8 wt%) containing 5 μg of SDF-1 (○) and empty gelatin hydrogels (△) or injection of SDF-1 solution (5 μg) (□). *: $p<0.05$, significant against the capillary number of SDF-1 solution group, †: $p<0.05$, significant against the capillary number of empty gelatin hydrogel group.

In addition to the electrostatic interaction, there are hydrophobic and hydrogen bonding interactions between SDF-1 and gelatin molecules. In the case of basic fibroblast growth factor (bFGF) of positive charge, the electrostatic interaction mainly plays an important role in the bFGF immobilization into the hydrogel for the release [15]. Since the SDF-1 molecule has the electric nature similar to bFGF, it is conceivable that the electrostatic interaction with gelatin is prominent for the SDF-1 release. The *in vitro* (Figure 1) and *in vivo* (Figure 2) conditions affect the release profile of SDF-1. In the

former, since PBS does not contain any enzymes to degrade the hydrogel, only the SDF-1 molecules in the free form can be released from the hydrogel. On the other hand, the *in vivo* system contains enzymes and proteins. It is likely that upon degradation of the hydrogel, the SDF-1 immobilized can be solubilized in water, resulting in the release from the hydrogel. Generally considering that the longer SDF-1 release results in the higher angiogenesis *in vivo*, it is practically reasonable to take the hydrogel system which can release *in vivo* for longer time periods. Based on this point, the succinylated gelatin hydrogel was used for *in vivo* angiogenic experiments. The crosslinking extent of succinylated gelatin hydrogels was changed by the preparation conditions (Table 3), and the time profiles of SDF-1 release and hydrogels degradation were greatly influenced by the crosslinking extent of hydrogels (Figures 3 and 4). The crosslink density of hydrogels (average water content: 95.8, 96.3, 97.9, and 98.8 wt%) estimated based on the Flory swelling equation [16] was 58, 42, 11, and 3.2 x 10^{-7}/cm^3 respectively. On the other hand, the molecular size of SDF-1 dimer is reported to be smaller than 7.1 nm in diameter [17]. Since the size is small enough compared with the network of hydrogels calculated from the crosslinking density, we can say that free SDF-1 molecule can easily pass through the water phase in hydrogel. Taken together, if the SDF-1 does not interact with the succinylated gelatin, it would be rapidly released out from the hydrogel. When the percent remaining of SDF-1 was compared, it tended to become higher with a decrease in the water content of hydrogels. This finding can be explained in terms of crosslinking extent by glutaraldehyde. The crosslinking reaction decreased the amino groups of gelatin and consequently the $COOH/NH_2$ ratio of gelatin would become larger. It is possible that the increased negative charge enhances the

interaction between the SDF-1 and gelatin, resulting in increased the percent remaining of SDF-1.

A good correlation of the *in vivo* time profile between the SDF-1 release and hydrogel degradation (Figure 5) clearly indicates that the SDF-1 release was not governed by the simple diffusion mechanism of SDF-1, but by the degradation of gelatin hydrogel. This phenomenon was identical with that of bFGF release reported previously [18]. Taken together, we could be confirmed that SDF-1 was released by weakening electrostatic interaction following release carrier degradation.

CXCR4 is an inherent receptor of SDF-1, which is expressed on hematopoietic cells, vascular endothelial cells, and mesenchymal stem cells [6, 19-24]. As shown in Figure 6, the expression of CXCR4 mRNA increased in the tissue around the implanted gelatin hydrogels containing SDF-1. The expression level compared with that of the positive control (thymus tissue) was depended on the SDF-1 implantation dose and the highest expression was observed at the dose of 5 μg. Taken together, it is highly possible that the implantation of SDF-1-incorporated gelatin hydrogels enhanced the accumulation of CXCR4 positive cells around the site implanted, resulting in the enhanced regeneration of capillary (Figures 7 and 8). Salcedo *et al*. [25] reported that angiogenesis was detected in mice after the local daily subcutaneous injection of 1μg SDF-1 four times repeatedly. However, the effective threshold dose of SDF-1 to induce angiogenesis is still unknown. There are other reports that 60 or 100 ng of SDF-1 dose was good for cell accumulation [26] or treatment of myocardiac infarction [27]. Further experiments should be needed to determine the optimal dose of SDF-1 implanted. In the range of lower SDF-1 doses, the level of CXCR4-positive cells accumulated around the

implanted site decreased with the dose, but higher SDF-1 doses decreased the level (Figure 6). This phenomenon can be explained in terms of the SDF-1 receptor down regulation. Generally, the chemokine action decreased when the dose is too high because of the down-regulation of the receptor [28]. An appropriate amount of SDF-1 released from the hydrogel would be effective in the mobilization of CXCR4-positive cells and consequently angiogenesis enhancement.

In vivo regeneration assay of capillary vessels showed that faster and stronger regeneration of new capillaries was detected for the hydrogel containing SDF-1 than for the empty hydrogel and SDF-1 solution. Succinylated gelatin hydrogel containing SDF-1 was used for the angiogenic assay in terms of positive cell accumulation of the receptor, CXCR4 because the maximum remaining of SDF-1 was observed after subcutaneous implantation with the hydrogel. It is possible that the gelatin hydrogel enabled to release SDF-1 locally in a controlled manner, resulting in an enhanced recruitment of cells for angiogenesis (Figures 7 and 8). Significantly higher number of capillaries regenerated was observed for the gelatin containing SDF-1 4 and 7 days after implantation than the case of the SDF-1-injected group. In addition, the mean number of capillaries seems to decrease for the gelatin containing SDF-1 10 days after treatment. However, no significant difference in the capillary number was observed among all the experimental groups 10 days after treatment. These phenomena were also observed in the study previously reported from Ichioka *et al.* [14]. It is known that necessary mediators are secreted to initiate and promote the wound repair process at normal murine skin after creation of defect. SDF-1 is also reported to express in the inflammation stage [29, 30]. It could be thought that the normal wound repair process

was accelerated by the implantation of gelatin hydrogels containing SDF-1. It is believed that this result strongly indicates feasibilities of the SDF-1-incorporated hydrogel in angiogenic induction. Many researchers have been reported on angiogenesis using vascular endothelial growth factor, FGF family, and other growth factors or chemokines [31-33]. It has been known that SDF-1 is induced by the factors, such as VEGF or bFGF, and plays an important role as a factor to recruit bone marrow-derived circulating cells [25, 34]. Considering the inherent nature of SDF-1, it is no doubt that the controlled release of SDF-1 is a promising strategy to induce angiogenesis by making use of the natural healing potential of living body.

There are several reports about the controlled release of SDF-1. Schantz *et al*. [26] examined the homing of mesenchymal stem cells by SDF-1 treatment with an infusion pump *in vivo*. The combination of vascular endothelial growth factor, SDF-1, and bone morphogenetic protein-6 (60 ng each/ten days infusion) was effective in inducing vascularization into a poly(caprolactone) scaffold. Zhang *et al.* [27] reported the controlled release of SDF-1 from poly(ethylene glycol) (PEG)-conjugated fibrin and the therapeutic effect with a myocardial infarction model. SDF-1 could be released from the fibrin matrix up to 10 days and the SDF-1 release could improve the symptom of acute myocardial infarction through an enhanced homing of c-kit$^+$ cells. Hiasa *et al*. reported the gene expression of SDF-1 by an electroporation method with the plasmid DNA at the site of myocardiac infarction [35]. The SDF-1 protein was expressed up to 14 days in the muscle and the good therapeutic efficacy resulted from the mobilization of endothelial progenitor cells into the infarction site. The effective use of SDF-1 enhances the recruitment of key cells into the site necessary, resulting in the cells-based

enhancement of angiogenesis thereat. To this end, it is necessary to develop the system for enhanced biological activity of SDF-1 *in vivo*. For tissue engineering and regenerative medicine, minimally invasive and low-risk treatments are required and should be developed. By our release system, only a single implantation of biodegradable hydrogel could enhance the natural healing potential of body, and consequently assist positively the healing process of diseases. The release profiles of SDF-1 could be modified by changing the crosslinking extent of hydrogels (Figure 4). The SDF-1 release technology will be available to treat other diseases. The combination with other therapeutic agents is another strategy to enhance the therapeutic efficacy of tissue regeneration.

REFERENCES

1. Langer R. Tissue Engineering: Perspectives, Challenges, and Future Directions. Tissue Eng 2007;13:1-2.
2. Kimura Y, Tabata Y. Experimental Tissue Regeneration by DDS Technology of Bio-Signaling Molecules. J Dermatol Sci 2007;47:189-99.
3. Marui A, Tabata Y, Kojima S, Yamamoto M, Tambara K, Nishina T, Saji Y, Inui K, Hashida T, Yokoyama S, Onodera R, Ikeda T, Fukushima M, Komeda M. A Novel Approach to Therapeutic Angiogenesis for Patients with Critical Limb Ischemia by Sustained Release of Basic Fibroblast Growth Factor Using Biodegradable Gelatin Hydrogel: An Initial Report of the Phase I-IIa Study. Circ J 2007;71:1181-6.
4. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The Pleiotropic Effects of the SDF-1-CXCR4 Axis in Organogenesis, Regeneration and Tumorigenesis. Leukemia 2006;20:1915-24.
5. Kollet O, Dar A, Lapidot T. The Multiple Roles of Osteoclasts in Host Defense: Bone Remodeling and Hematopoietic Stem Cell Mobilization. Annu Rev Immunol 2006;25:51-69.
6. Lapidot T, Dar A, Kollet O. How Do Stem Cells Find Their Way Home? Blood 2005;106:1901-10.
7. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the Stromal Cell-Derived Factor-1/CXCR4 Pathway in Prostate Cancer Metastasis to Bone. Cancer Res 2002;62:1832-7.
8. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H,

Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-Mediated Deployment of SDF-1 Induces Revascularization through Recruitment of CXCR4+ Hemangiocytes. Nat Med 2006;12:557-67.

9. Hori K, Sotozono C, Hamuro J, Yamasaki K, Kimura Y, Ozeki M, Tabata Y, Kinoshita S. Controlled-Release of Epidermal Growth Factor from Cationized Gelatin Hydrogel Enhances Corneal Epithelial Wound Healing. J Control Release 2007;118:169-76.
10. C.o.M.f.T.P. Gelatin, PAGI (Photographic and Gelatin Industries) Method, 9th Ed., 2002.
11. Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In Vitro Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials 1998;19:1781-9.
12. Ozeki M, Tabata Y. In Vivo Degradability of Hydrogels Prepared from Different Gelatins by Various Cross-Linking Methods. J Biomater Sci Polym Ed 2005;16:549-61.
13. Bolton AE, Hunter WM. The Labelling of Proteins to High Specific Radioactivities by Conjugation to a ^{125}I-Containing Acylating Agent. Biochem J 1973;133:529-39.
14. Ichioka S, Kouraba S, Sekiya N, Ohura N, Nakatsuka T. Bone Marrow-Impregnated Collagen Matrix for Wound Healing: Experimental Evaluation in a Microcirculatory Model of Angiogenesis, and Clinical Experience. Br J Plast Surg 2005;58:1124-30.
15. Ikada Y, Tabata Y. Protein Release from Gelatin Matrices. Adv Drug Deliv Rev 1998;31:287-301.

16. Park H, Park K, Shalaby W. Biodegradable Hydrogels for Drug Delivery. Lancaster, PA: Technomic Publishing, 1993.
17. Ryu EK, Kim TG, Kwon TH, Jung ID, Ryu D, Park YM, Kim J, Ahn KH, Ban C. Crystal Structure of Recombinant Human Stromal Cell-Derived Factor-1alpha. Proteins 2007;67:1193-7.
18. Tabata Y, Ikada Y. Vascularization Effect of Basic Fibroblast Growth Factor Released from Gelatin Hydrogels with Different Biodegradabilities. Biomaterials 1999;20:2169-75.
19. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The Hiv Coreceptors Cxcr4 and Ccr5 Are Differentially Expressed and Regulated on Human T Lymphocytes. Proc Natl Acad Sci U S A 1997;94:1925-30.
20. Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. Chemokine Receptors in Human Endothelial Cells. Functional Expression of CXCR4 and Its Transcriptional Regulation by Inflammatory Cytokines. J Biol Chem 1998;273:4282-7.
21. Horuk R. Chemokine Receptors. Cytokine Growth Factor Rev 2001;12:313-35.
22. Jo DY, Rafii S, Hamada T, Moore MA. Chemotaxis of Primitive Hematopoietic Cells in Response to Stromal Cell-Derived Factor-1. J Clin Invest 2000;105:101-11.
23. Kortesidis A, Zannettino A, Isenmann S, Shi S, Lapidot T, Gronthos S. Stromal-Derived Factor-1 Promotes the Growth, Survival, and Development of Human Bone Marrow Stromal Stem Cells. Blood 2005;105:3793-801.
24. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The Chemokine Receptor CXCR-4 Is Expressed on CD34+ Hematopoietic Progenitors and Leukemic Cells and Mediates Transendothelial Migration Induced by Stromal

Cell-Derived Factor-1. Blood 1998;91:4523-30.

25. Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ. Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor Induce Expression of CXCR4 on Human Endothelial Cells: In Vivo Neovascularization Induced by Stromal-Derived Factor-1alpha. Am J Pathol 1999;154:1125-35.
26. Schantz JT, Chim H, Whiteman M. Cell Guidance in Tissue Engineering: SDF-1 Mediates Site-Directed Homing of Mesenchymal Stem Cells within Three-Dimensional Polycaprolactone Scaffolds. Tissue Eng 2007;13:2615-24.
27. Zhang G, Nakamura Y, Wang X, Hu Q, Suggs LJ, Zhang J. Controlled Release of Stromal Cell-Derived Factor-1alpha in Situ Increases c-kit+ Cell Homing to the Infarcted Heart. Tissue Eng 2007;13:2063-71.
28. Pelchen-Matthews A, Signoret N, Klasse PJ, Fraile-Ramos A, Marsh M. Chemokine Receptor Trafficking and Viral Replication. Immunol Rev 1999;168:33-49.
29. Havens AM, Chiu E, Taba M, Wang J, Shiozawa Y, Jung Y, Taichman LS, D'Silva NJ, Gopalakrishnan R, Wang C, Giannobile WV, Taichman RS. Stromal-Derived Factor-1alpha (CXCL12) Levels Increase in Periodontal Disease. J Periodontol 2008;79:845-53.
30. Jiang HW, Ling JQ, Gong QM. The Expression of Stromal Cell-Derived Factor 1 (SDF-1) in Inflamed Human Dental Pulp. J Endod 2008;34:1351-4.
31. Bussolino F, Albini A, Camussi G, Presta M, Viglietto G, Ziche M, Persico G. Role of Soluble Mediators in Angiogenesis. Eur J Cancer 1996;32A:2401-12.
32. Folkman J. Angiogenesis: An Organizing Principle for Drug Discovery? Nat Rev

Drug Discov 2007;6:273-86.

33. Tabata Y. Significance of Release Technology in Tissue Engineering. Drug Discov Today 2005;10:1639-46.
34. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Yung S, Chimenti S, Landsman L, Abramovitch R, Keshet E. VEGF-Induced Adult Neovascularization: Recruitment, Retention, and Role of Accessory Cells. Cell 2006;124:175-89.
35. Hiasa K, Ishibashi M, Ohtani K, Inoue S, Zhao Q, Kitamoto S, Sata M, Ichiki T, Takeshita A, Egashira K. Gene Transfer of Stromal Cell-Derived Factor-1alpha Enhances Ischemic Vasculogenesis and Angiogenesis Via Vascular Endothelial Growth Factor/Endothelial Nitric Oxide Synthase-Related Pathway: Next-Generation Chemokine Therapy for Therapeutic Neovascularization. Circulation 2004;109:2454-61.

Chapter 7

Regeneration of bone tissue by gelatin hydrogels containing bone morphogenetic protein (BMP)-2

INTRODUCTION

Tissue engineering has been extensively studied for about twenty years. The strategy needs precursor cells, the scaffolds for cells attachment, and bio-signaling molecules of growth factors. Especially, the precursor or stem cells, like embryonic stem cells or induced pluripotent stem cells, have been studied, and some mechanisms of their differentiation into specific cell lineages have been clarified recently [1, 2]. From the mechanisms well recognized, several soluble factors bind to the cell receptor and subsequently transmit the molecular signals for the specific gene expression. In addition, it has been demonstrated that the matrix present around cells, called the extracellular matrix (ECM), plays an important role to enhance the signals and their biological functions [3-5].

Recent research reports have strongly suggested that stem cells circulating in the blood and body are originally present for hematopoiesis, vascularization or mesenchymal tissue regeneration [6-14]. For example, a promoted recruitment of endogenous cells to a site would result in the cell-based tissue regeneration at the site.

Therefore, if the *in vivo* fate of the cells can be regulated by the implantation of materials, the endogenous cell-based tissue regeneration can be achieved. For example, Tabata *et al.* have developed gelatin hydrogels for the controlled release of various bio-signaling molecules, such as growth factors, chemokines, and genes [15]. Among them, bone morphogenetic protein (BMP)-2 is known as a strong inducer of bone tissue regeneration [16]. In addition, it is reported that BMP-2 has an activity to promote the recruitment of cells [17]. This activity is promising from the viewpoint of tissue regeneration induced by cells originally present in the body. In this study, the gelatin hydrogel was prepared for the controlled release of BMP-2. After the hydrogel containing BMP-2 was implanted subcutaneously, the ectopic bone regeneration was evaluated by radiological and histological examinations. The recruitment of bone marrow-derived osteoblast progenitor cells at the site of BMP-2 released was examined.

EXPERIMENTAL

Materials

A gelatin sample with an isoelectric point (IEP) of 9.0 was prepared from the porcine skin collagen (Nitta Gelatin Co., Osaka, Japan) by an acidic treatment. Glutaraldehyde (GA), glycine, and other chemicals were obtained from Wako Pure Chemical Industries, Osaka, Japan and used without further purification.

Preparation of gelatin hydrogels

Chemically-crosslinked gelatin hydrogels with glutaraldehyde (GA) were prepared according to the method previously reported [18]. Briefly, aqueous solution of 3 wt% gelatin (pH 5.0) was mixed with GA at a final concentration of 0.16 or 0.09 %(w/v) respectively, followed by leaving at 4 °C for 12 hr for gelatin crosslinking. The gelatin hydrogel crosslinked was treated by 0.1 M glycine solution to block the residual aldehyde groups. After washing three times with double-distilled water (DDW), the hydrogels were freeze-dried. The crosslinking extent of hydrogels prepared was evaluated by measuring the water content. The water contents of hydrogels prepared at the higher and lower GA concentrations were 97.5 $\pm$ 0.1 or 99.3 $\pm$ 0.0 wt%, respectively. The previous study revealed that the *in vivo* time periods of hydrogels degradation were approximately 4-5 and 2-3 weeks, respectively [18].

Preparation of GFP-chimeric mice

All the surgical and observation procedures were performed under continuous inhalation anesthesia by isoflurane (Forane®, Abbott Japan Ltd., Osaka, Japan) with 400 anesthesia unit (Univentor Ltd., Zejtun, Malta). C57BL/6 transgenic mice that ubiquitously express enhanced green fluorescent protein (GFP), were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Preparation of chimeric mice was performed according to the procedure previously reported [20]. Briefly, bone marrow cells were isolated from 8- to 10-week-old male transgenic mice under sterile conditions [21]. Eight- to 10-week-old female C57BL/6 mice were irradiated lethally with 10 Gy of gamma-ray. For the total bone marrow

transplantation (BMT), 5 x 10^6 of bone marrow cells prepared from GFP transgenic mice was administered to recipient mice irradiated. After the transplantation, the mice were bred for 10 weeks to complete the replacement of bone marrow cells to GFP-positive cells. The replacement ratio of bone marrow cells was 93.2 $\pm$ 1.5 % when evaluated by the flow cytometry (FACSCalibur, BD Bioscience, Franklin Lakes, NJ).

***In vivo* regeneration assay of bone tissue**

Human recombinant BMP-2 (Yamanouchi Pharmaceutical Co., Tokyo, Japan) was dissolved in 10 mM of phosphate-buffered saline solution (PBS, pH 7.4) at 100 μg/ml. The solution (30 μl) was dropped on the gelatin hydrogel (2 mg) to allow it to swell into the hydrogel. After incubation of the hydrogels containing BMP-2 at 4 °C for 12 hr, the hydrogels were implanted to the back subcutis of GFP-chimeric mice. As a control, gelatin hydrogels containing PBS were similarly implanted to the back of the mice. Then, the tissue around the sites implanted was extracted at different time intervals after hydrogel implantation, and the fluorescent images of tissues were obtained by a digital microscope (Multiviewer system VB-S20; Keyence, Osaka, Japan). Bone regeneration was radiologically examined by a soft x-ray machine (Hitex-100, Hitachi, Japan) at 54 kV and 2.5 mA for 20 sec. Following the tissues extracted were fixed with 4 % paraformaldehyde at 4 °C for 48 hr, the bone tissue were decalcified with ethylenediamine tetraaceticacid disodium salt (EDTA) solution at 4 °C for 6 days. The EDTA solution was changed every other day. After decalcification, the pellets were equilibrated in PBS containing 15 wt% sucrose for 12 hr and then in PBS containing 30 wt% sucrose for 12 hr, embedded in Tissue-Tek OCT Compound (Sakura Finetek,

Tokyo, Japan), frozen on dry ice, and stored at -80 °C. For the histological examinations, the 6-μm-thick sections were cut with a cryostat (Leica Microsystems AG, Wetzlar, Germany) at the portion of implanted site as central as possible, followed by staining with hematoxylin and eosin (HE). The area of bone tissue regenerated was assessed in terms of histological image analysis using a computer program of Image-Pro Plus 3.01 (Media-Cybernetics, Silver Spring, MD).

Immunofluorescence staining

After PBS washing, the sections (6 μm thickness) blocked with a normal goat serum for 1 hr at room temperature before incubation with a polyclonal anti-mouse osteocalcin antibody (1:250; Takara Bio, Shiga, Japan) for 1 hr at room temperature. Then, the sections were stained with a TRITC-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 hr at room temperature. After washing with PBS, the sections were mounted with Vectashield® mounting medium (Vector Laboratories, Burlingame, CA). The fluorescence images were obtained on an epifluorescent microscope (AX-80, Olympus Co. Tokyo, Japan), while the percentage GFP-positive cells to osteocalcin-positive cells in each image was determined from the images. Three areas interested (100 x 100 μm^2) were chosen randomly from each fluorescence image (at least 4 images per each experimental group) and the number of GFP- and osteocalcin-positive cells was counted.

***In vitro* migration assay**

Bone marrow cells isolated from GFP transgenic mice described above were

cultured to expand in minimum essential medium alpha (αMEM, Sigma-Aldrich, St. Louis, MO) containing 15 %(v/v) fetal bovine serum (FBS), and the medium was changed to αMEM without serum 24 hr before the migration assay experiment. The cells were trypsinized and plated onto the HTS® fluoroblok inserts (Falcon #351552 with 8 μm diameter pore, Becton Dickinson, Franklin Lakes, NJ) with αMEM containing 0.5 %(v/v) FBS. The bottom side of the inserts was contacted to αMEM containing 15 or 0.5 %(v/v) FBS, 100 ng/ml of recombinant human stromal cell-derived factor-1 (SDF-1, #350-NS/CF, R&D systems Inc. Minneapolis, MN), BMP-2, or recombinant human placental growth factor (PlGF, #264-PG, R&D systems) with 0.1 %(v/v) bovine serum albumin. After 24 hr culture, cells migrated to the bottom side were counted from fluorescence photographs taken by an epifluorescent microscope (IX-70, Olympus). The number of cells in 6 images (0.594 mm^2 per each image) was counted.

Statistical analysis

All the results were statistically analyzed by the unpaired student's t test. $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean + the standard deviation of the mean (SD).

RESULTS

Bone regeneration by gelatin hydrogels containing BMP-2

Figure 1 shows the soft X-ray radiophotographs of the sites implanted two weeks after the implantation of gelatin hydrogels containing 3 μg of BMP-2 or PBS. A radioopacity was observed in the center of tissues implanted with gelatin hydrogels containing BMP-2, although the influence of the water content on the extent was not observed. On the contrary, no radioopacity was observed for the BMP-2-free gelatin hydrogels.

Figure 2 shows the HE picture of site implanted 7 weeks after implantation of the gelatin hydrogel containing 3 μg of BMP-2. After implantation, mature bone tissues with bone marrow-like structure were observed. The gelatin hydrogels implanted was completely degraded. Figure 3 shows the area of bone tissue regenerated at the site implanted with gelatin hydrogels containing BMP-2. After the implantation of gelatin hydrogel containing BMP-2 with a water content of 99.3 %, the regeneration of bone tissue was observed only at 2 weeks, but thereafter the tissue regenerated disappeared. On the contrary, the implantation of hydrogels containing BMP-2 with a water content of 97.5 % induced significantly the bone tissue regeneration and the tissue retained even 7 weeks after the implantation.

Recruitment of cells by gelatin hydrogels containing BMP-2

Figure 4 shows the fluorescence images of tissues around implanted sites 2 weeks after the implantation of gelatin hydrogels containing 3 μg of BMP-2 or PBS.

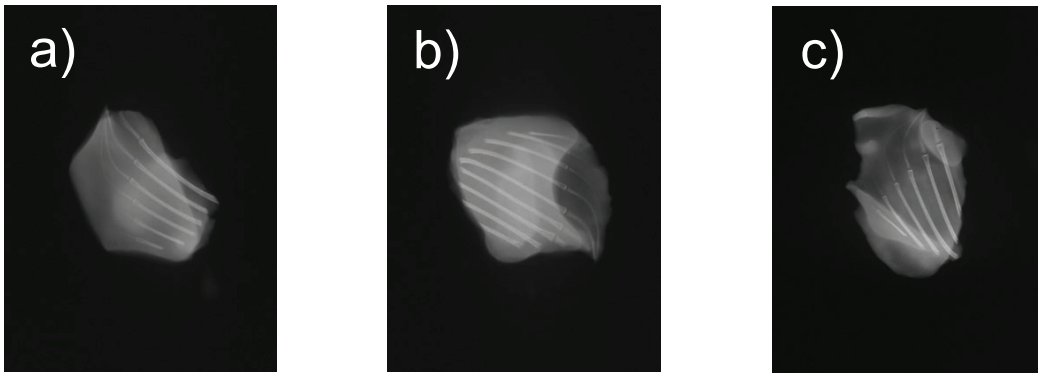

Figure 1. Radiophotography of tissues around the site implanted 2 weeks after implantation of (a) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 97.5 %, (b) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 99.3 %, and (c) the gelatin hydrogel containing PBS with a water content of 97.3 %.

Irrespective of the experimental groups, the sites implanted had a green fluorescence, which indicates the accumulation of bone marrow-derived cells.

Figure 5 shows the immunofluorescence images of tissues around the site implanted 2 weeks after implantation of gelatin hydrogels containing 3 μg of BMP-2 or PBS. For gelatin hydrogels containing BMP-2, many red-stained cells were observed around the site implanted (Figures 5a and 5b). On the contrary, no cells were observed around the site implanted with gelatin hydrogels (Figure 5c). Figure 6 shows the percentage of GFP-positive cells to osteocalcin-positive cells around the site implanted 2 weeks after implantation of gelatin hydrogels containing 3 μg of BMP-2 or PBS. The implantation of gelatin hydrogels containing BMP-2 with both the water contents, increased the percentage of GFP-positive cells to osteocalcin-positive cells around the site implanted, although the percentage for the gelatin hydrogel containing BMP-2 with a water content of 97.5 % was significantly higher than that of hydrogels with a water

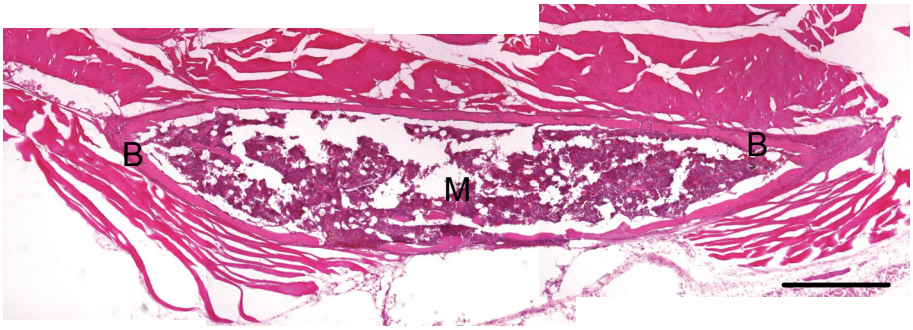

Figure 2. Histological section of tissues around the site implanted 7 weeks after implantation of gelatin hydrogel containing BMP-2 (3 μg) with water content of 97.5 %. B: bone tissue, M: bone marrow-like structure. (HE staining, Scale bar = 500 μm)

content of 99.3 %.

***In vitro* cell migration**

Figure 7 shows the number of migrated cells through the transwell membrane 24 hr after incubation with αMEM containing BMP-2 and other factors. No chemoattractant activity toward bone marrow cells was observed for BMP-2, similar to the negative control [0.5 %(v/v) FBS]. However, both SDF-1 and PlGF had a strong chemoattractant activity to the same level as the positive control [15 %(v/v) FBS]. The activity of PlGF was significantly higher than that of SDF-1 and 15 %(v/v) FBS.

DISCUSSION

This study demonstrates that BMP-2 release profile affects the extent of bone marrow-derived cells accumulated and the consequent regeneration of bone tissues. The

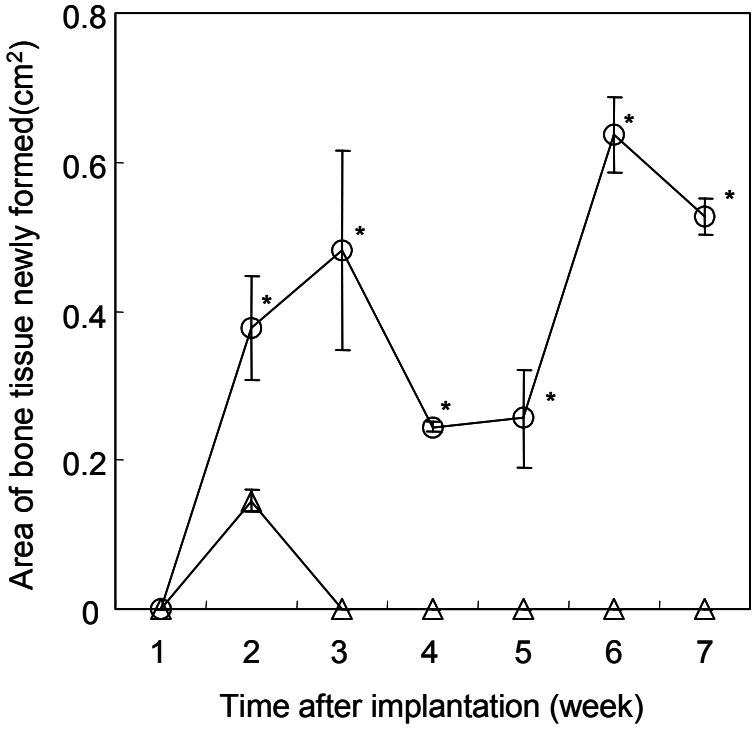

Figure 3. Area of bone tissues regenerated after implantation of (○) gelatin hydrogels containing BMP-2 (3 μg) with a water content of 97.5 %, (△) gelatin hydrogels containing BMP-2 (3 μg) with a water content of 99.3 %. *: $p < 0.05$, significant against the area after implantation of gelatin hydrogels containing BMP-2 (3 μg) with a water content of 99.3 %.

BMP-2 release for a longer time period enabled the strong accumulation of GFP-positive bone marrow-derived osteoblast progenitor cells which are also stained with the anti-osteocalcin antibody, even 2 weeks after the implantation. It is apparent from Figure 4 that the accumulation of bone marrow-derived cells was observed by the implantation of gelatin hydrogels with or without BMP-2 incorporating. However, from the double staining assay, for the hydrogel without BMP-2, no-osteocalcin-positive cells were detected around the implanted site (Figure 6). Since the osteocalcin-expressed cells are generally osteoblastic cells with a bone regeneration activity, we can say with

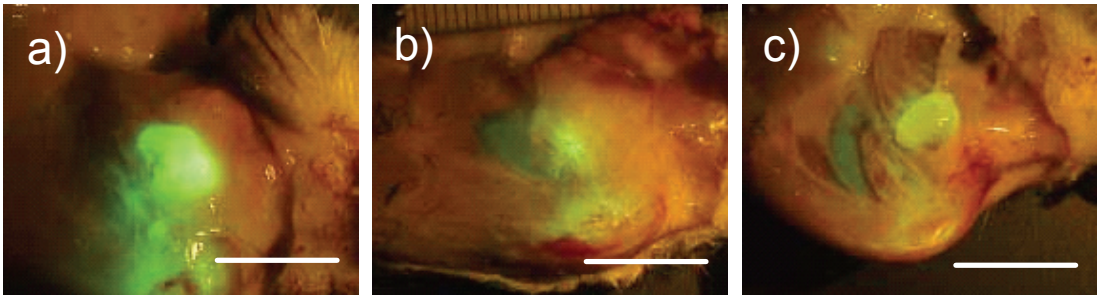

Figure 4. Fluorescence images around the site implanted 2 weeks after implantation of (a) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 97.5 %, (b) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 99.3 %, and (c) the gelatin hydrogel containing PBS with a water content of 97.3 %. Scale bar = 1 cm.

certainty that the BMP-2 release increased the recruitment of osteoblastic cells around the site released. It is reported that cells are promoted to recruit to the site of BMP-2 presence [17].

The extent of bone tissue regeneration depended on the water contents of gelatin hydrogels. This finding is experimentally confirmed by the previous study [18]. The decrease in the area regenerated was observed in 4 or 5 and 3 weeks after implantation of gelatin hydrogels with the water contents of 97.5 and 99.3 %, respectively. This time profile can be explained in terms of that of BMP-2 release. For the gelatin hydrogel, the time profile of BMP-2 release is well correlated to that of hydrogel degradation. The hydrogel which is degraded for 4-5 weeks would release BMP-2 for 4-5 weeks. It is possible that for this range, the BMP-2 release result in the BMP-induced regeneration of bone tissue. However, termination of the release suppresses the bone tissue induction, resulting in the consequent bone tissue disappearance. The area of bone regenerated increased again 6 weeks later. The cells

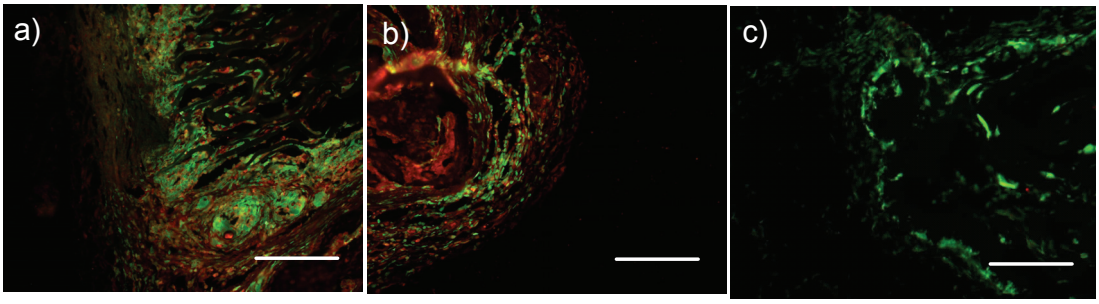

Figure 5. Immunofluorescence staining images of tissue around the site implanted 2 weeks after implantation of (a) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 97.5 %, (b) the gelatin hydrogel containing BMP-2 (3 μg) with a water content of 99.3 %, and (c) the gelatin hydrogel containing PBS with a water content of 97.3 %. Red fluorescence: osteocalcin, Green fluorescence: GFP. Scale bar = 200 μm.

recruited by BMP-2 released and remained may function to further promote bone regeneration.

Significant difference in the accumulation of osteocalcin-positive cells between the gelatin hydrogels with water contents of 97.5 and 99.3 % was observed (Figure 6). The present experiment revealed that the profile of BMP-2 release affects the recruitment of bone marrow-derived cells. It is conceivable that BMP-2 release for a longer time period induces the recruitment of cells for a long time period, resulting in enhanced accumulation of cells. BMP-2 has many activities for regeneration of bone tissues [22], such as osteoblast migration [23], promoting osteogenic differentiation of mesenchymal stem cells [24, 25], angiogenesis [26], apoptosis of osteoblast [27], and recruitment of osteoblast progenitor cells [17, 20]. Several studies recently reported that BMP-2 could induce the expression of PlGF. The enhanced expression contributed to the recruitment of the progenitor cells from the bone marrow [28-30]. The

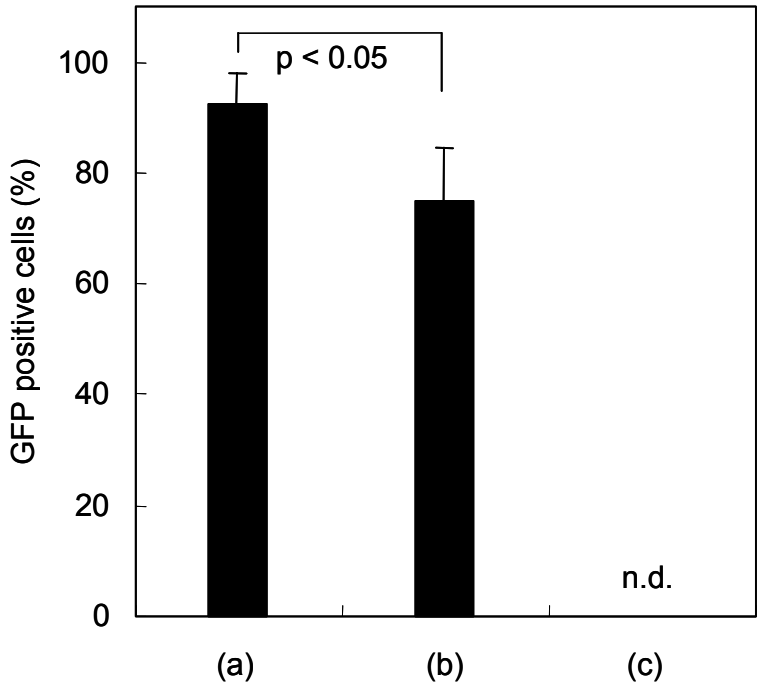

Figure 6. The percentage of GFP-positive cells to osteocalcin-positive cells around the site implanted 2 weeks after implantation of (a) gelatin hydrogels containing BMP-2 (3 μg) with a water content of 97.5 %, (b) gelatin hydrogels containing BMP-2 (3 μg) with a water content of 99.3 %, and (c) gelatin hydrogels containing PBS with a water content of 97.5 %. n.d.: not detected.

chemoattractant study revealed that SDF-1 and PlGF had a strong activity to accelerate the migration of bone marrow cells isolated, but BMP-2 itself did not have the activity (Figure 7). As one possibility, BMP-2 functions as an expression trigger of homing factors of SDF-1 or PlGF for bone marrow-derived cells. Further analysis should be needed to understand the effect of BMP-2 release on the cell recruitment.

This chapter clearly demonstrates that the BMP-2 releasing material enhances cell accumulation for regeneration of bone tissue. This activity could be modified by the release profile. The present finding opens a new strategy of tissue

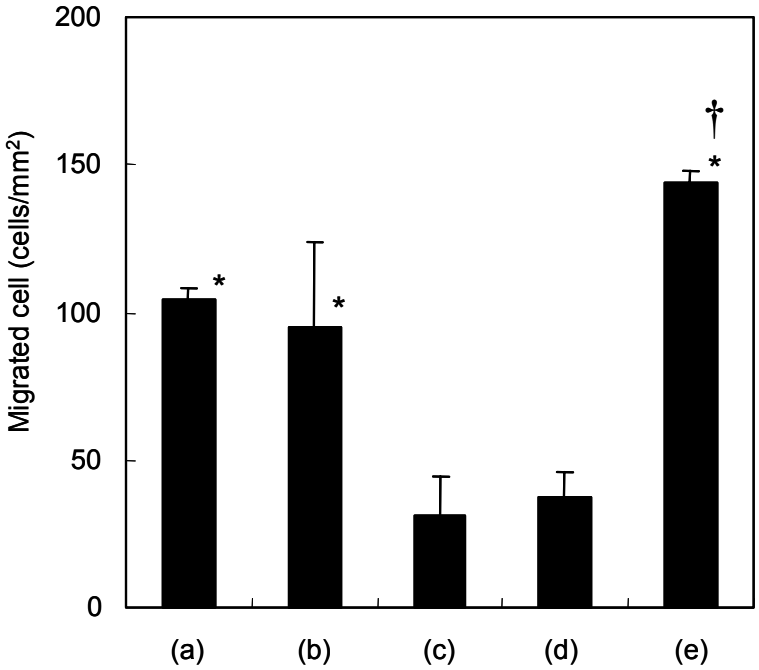

Figure 7. Migration of bone marrow cells through the transwell membrane 24 hr after incubation with αMEM containing (a) 15 %(v/v) FBS, (b) 100 ng/ml SDF-1, (c) 100 ng/ml BMP-2, (d) 0.5 %(v/v) FBS, and (e) 100 ng/ml PlGF. *: $p < 0.05$ against the groups (c) and (d), †: $p < 0.05$ significant against the groups (a) and (b).

engineering to achieve tissue regeneration without cell transplantation.

REFERENCES

1. Langer R. Tissue Engineering: Perspectives, Challenges, and Future Directions. Tissue Eng 2007;13:1-2.
2. Morrison SJ, Spradling AC. Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance Throughout Life. Cell 2008;132:598-611.
3. Ingber DE, Mow VC, Butler D, Niklason L, Huard J, Mao J, Yannas I, Kaplan D, Vunjak-Novakovic G. Tissue Engineering and Developmental Biology: Going Biomimetic. Tissue Eng 2006;12:3265-83.
4. Matsumoto T, Mooney DJ. Cell Instructive Polymers. Adv Biochem Eng Biotechnol 2006;102:113-37.
5. Badylak SF. The Extracellular Matrix as a Biologic Scaffold Material. Biomaterials 2007;28:3587-93.
6. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor Cell Trafficking Is Regulated by Hypoxic Gradients through Hif-1 Induction of SDF-1. Nat Med 2004;10:858-64.
7. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating Osteoblast-Lineage Cells in Humans. N Engl J Med 2005;352:1959-66.
8. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Yung S, Chimenti S, Landsman L, Abramovitch R, Keshet E. VEGF-Induced Adult Neovascularization: Recruitment, Retention, and Role of Accessory Cells. Cell 2006;124:175-89.
9. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H,

Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-Mediated Deployment of SDF-1 Induces Revascularization through Recruitment of Cxcr4+ Hemangiocytes. Nat Med 2006;12:557-67.

10. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating Skeletal Stem Cells. J Cell Biol 2001;153:1133-40.
11. Wan C, He Q, Li G. Allogenic Peripheral Blood Derived Mesenchymal Stem Cells (MSCs) Enhance Bone Regeneration in Rabbit Ulna Critical-Sized Bone Defect Model. J Orthop Res 2006;24:610-8.
12. Wragg A, Mellad JA, Beltran LE, Konoplyannikov M, San H, Boozer S, Deans RJ, Mathur A, Lederman RJ, Kovacic JC, Boehm M. VEGFR1/CXCR4-Positive Progenitor Cells Modulate Local Inflammation and Augment Tissue Perfusion by a SDF-1-Dependent Mechanism. J Mol Med 2008;86:1221-32.
13. Zhou B, Han ZC, Poon MC, Pu W. Mesenchymal Stem/Stromal Cells (MSC) Transfected with Stromal Derived Factor 1 (SDF-1) for Therapeutic Neovascularization: Enhancement of Cell Recruitment and Entrapment. Med Hypotheses 2007;68:1268-71.
14. Zhu W, Boachie-Adjei O, Rawlins BA, Frenkel B, Boskey AL, Ivashkiv LB, Blobel CP. A Novel Regulatory Role for Stromal-Derived Factor-1 Signaling in Bone Morphogenic Protein-2 Osteogenic Differentiation of Mesenchymal C2C12 Cells. J Biol Chem 2007;282:18676-85.
15. Tabata Y. Significance of Release Technology in Tissue Engineering. Drug Discov Today 2005;10:1639-46.

16. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel Regulators of Bone Formation: Molecular Clones and Activities. Science 1988;242:1528-34.
17. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Bone Marrow-Derived Osteoblast Progenitor Cells in Circulating Blood Contribute to Ectopic Bone Formation in Mice. Biochem Biophys Res Commun 2007;354:453-8.
18. Yamamoto M, Takahashi Y, Tabata Y. Controlled Release by Biodegradable Hydrogyels Enhances the Ectopic Bone Formation of Bone Morphogenetic Protein. Biomaterials 2003;24:4375-83.
19. Tabata Y, Nagano A, Muniruzzaman M, Ikada Y. In Vitro Sorption and Desorption of Basic Fibroblast Growth Factor from Biodegradable Hydrogels. Biomaterials 1998;19:1781-9.
20. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating Bone Marrow-Derived Osteoblast Progenitor Cells Are Recruited to the Bone-Forming Site by the CXCR4/Stromal Cell-Derived Factor-1 Pathway. Stem Cells 2008;26:223-34.
21. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green Mice' as a Source of Ubiquitous Green Cells. FEBS Lett 1997;407:313-9.
22. Wozney JM. Overview of Bone Morphogenetic Proteins. Spine 2002;27:S2-8.
23. Sotobori T, Ueda T, Myoui A, Yoshioka K, Nakasaki M, Yoshikawa H, Itoh K. Bone Morphogenetic Protein-2 Promotes the Haptotactic Migration of Murine Osteoblastic and Osteosarcoma Cells by Enhancing Incorporation of Integrin Beta1 into Lipid Rafts. Exp Cell Res 2006;312:3927-38.

24. Wagner TU. Bone Morphogenetic Protein Signaling in Stem Cells--One Signal, Many Consequences. Febs J 2007;274:2968-76.
25. Bessa PC, Pedro AJ, Klosch B, Nobre A, van Griensven M, Reis RL, Casal M. Osteoinduction in Human Fat-Derived Stem Cells by Recombinant Human Bone Morphogenetic Protein-2 Produced in Escherichia Coli. Biotechnol Lett 2008;30:15-21.
26. Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE, Lowik CW. Bone Morphogenetic Proteins Stimulate Angiogenesis through Osteoblast-Derived Vascular Endothelial Growth Factor A. Endocrinology 2002;143:1545-53.
27. Hay E, Lemonnier J, Fromigue O, Marie PJ. Bone Morphogenetic Protein-2 Promotes Osteoblast Apoptosis through a Smad-Independent, Protein Kinase C-Dependent Signaling Pathway. J Biol Chem 2001;276:29028-36.
28. Marrony S, Bassilana F, Seuwen K, Keller H. Bone Morphogenetic Protein 2 Induces Placental Growth Factor in Mesenchymal Stem Cells. Bone 2003;33:426-33.
29. Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, Gopalakrishnan R, Moermans K, Smets N, Verfaillie CM, Carmeliet P, Bouillon R, Carmeliet G. Placental Growth Factor Mediates Mesenchymal Cell Development, Cartilage Turnover, and Bone Remodeling During Fracture Repair. J Clin Invest 2006;116:1230-42.
30. Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE. VEGF-A and PlGF-1 Stimulate Chemotactic Migration of Human Mesenchymal Progenitor Cells.

Biochem Biophys Res Commun 2005;334:561-8.

SUMMARY

Chapter 1

Gelatin microspheres containing basic fibroblast growth factor (bFGF) were prepared for the controlled release of bFGF. Co-implantation with the gelatin microspheres enabled preadipocytes to induce adipose tissue regeneration at the implanted site. Preadipocytes isolated from human fat tissue were suspended with the gelatin microspheres containing bFGF and incorporated into a collagen sponge of cell scaffold. Following subcutaneous implantation of the collagen sponge incorporating human preadipocytes, and gelatin microspheres containing 1 μg of bFGF into the back of nude mice, adipose tissue was formed at the implanted site of collagen sponge within 6 weeks postoperatively although the extent depended on the number of preadipocytes transplanted and the bFGF dose. The regeneration of adipose tissue was significant compared with the implantation of collagen sponge incorporating human preadipocytes and 1μg of free bFGF. The area of adipose tissue regenerated was increased with the number of preadipocytes transplanted until to 1.0×10^5 cells/site and thereafter leveled off. The maximum area was observed at the bFGF dose of 1μg/site. The area was significantly smaller at the bFGF dose of 0.5 μg/site or larger than 1 μg/site. Immunohistochemical examination indicated that the adipose tissue regenerated was composed of human matured adipocytes. No adipogenesis was observed at the implanted site of collagen sponge incorporating either gelatin microspheres containing bFGF or human preadipocytes and the mixed gelatin microspheres containing bFGF and human preadipocytes. We conclude that combination of gelatin microspheres containing

bFGF and preadipocytes with the collagen sponge is essential to achieve tissue engineering of fat tissue.

Chapter 2

In vivo adipose tissue regeneration by preadipocytes was evaluated by combining with collagen sponges with different biodegradabilities and gelatin microspheres containing basic fibroblast growth factor (bFGF) for the controlled release. The sponge biodegradability was regulated from one week to four weeks by changing the crosslinking conditions in sponge preparation. The time profile of bFGF release could be controlled from one week to five weeks by the biodegradability of gelatin microspheres used. After combined with human preadipocytes and gelatin microspheres containing bFGF, the collagen sponges were implanted into the back subcutis of nude mice to evaluate the adipose tissue regeneration. The regeneration of adipose tissue was observed for every sponge. The area of adipose tissue regenerated became maximum for the collagen sponge with degradation time of two weeks. No influence of the bFGF release profile on the tissue area was detected.

Chapter 3

Proliferation and adipogenic differentiation of human preadipocytes were evaluated on cell culture plates coated type I collagen combined with extracellular matrix (ECM) components, such as type IV collagen, gelatin, hyaluronic acid, and laminin-1. A synergistic enhancement of preadipocyte proliferation was observed in culturing on the plates coated with the mixture of type IV and type I collagen.

Adipogenic differentiation was synergistically enhanced for preadipocytes cultured on the substrates coated with the mixture of type IV and type I collagen or laminin-1 and type I collagen. The results indicate that the basement membrane components of type IV collagen and laminin-1 could enhance the biological activity of human preadipocytes for adipogenic differentiation.

Chapter 4

Controlled release of basic fibroblast growth factor (bFGF) from gelatin microspheres achieved *de novo* adipogenesis at the implanted site of a basement membrane extract (Matrigel®). Following subcutaneous co-implantation of Matrigel® and gelatin microspheres containing 0.1 μg of bFGF into the back of mice, adipose tissue was formed at the implanted site after 4 weeks postoperatively although the extent increased with implantation time. Regeneration of adipose tissue was significantly faster than the co-implantation of Matrigel®, and 0.1 μg of free bFGF while a larger volume of the adipose tissue formed was retained 15 weeks later. When measured in Matrigel® co-implanted with the gelatin microspheres containing bFGF, the number of cells infiltrated into Matrigel® increased to a significantly high extent compared with the bFGF co-implantation. Matrigel® alone was much less effective in inducing regeneration of adipose tissue. We conclude that gelatin microspheres containing bFGF enable Matrigel® to efficiently induce *de novo* adipogenesis at the implanted site in respect to the regeneration rate and volume of adipose tissue.

Chapter 5

This study is an investigation to evaluate *in situ* adipose tissue regeneration in fat tissues. Gelatin microspheres with different water contents were prepared for the controlled release of basic fibroblast growth factor (bFGF). After a collagen sponge scaffold was incorporated by the microspheres containing 0, 0.01, 0.1, 1, and 10 μg of bFGF with or without syngeneic rat preadipocytes (1×10^5 cells/site) into a defect of rat fat tissue, adipogenesis at the implanted site of scaffold was evaluated histologically. *In situ* regeneration of adipose tissue accompanied with angiogenesis was observed in the scaffold implanted with the microspheres containing 1.0 μg of bFGF, although the extent was less at the lower and higher bFGF doses. The *in situ* regeneration induced by the microspheres containing bFGF was significantly higher than that induced by free bFGF of the same dose. Adipogenesis was enhanced with time after implantation up to 4 weeks and thereafter leveled off. Such *in situ* adipogenesis was reproducibly induced by implantation of collagen scaffold incorporating gelatin microspheres containing 1 μg of bFGF, whereas addition of rat syngeneic preadipocytes did not promote the adipogenesis. The degradation of microspheres and the consequent FGF release became faster with an increase in the water content of gelatin microspheres. Less *in situ* regeneration of adipose tissue was observed at the lower water content of microspheres, which showed longer-term bFGF release. We conclude that combination of scaffold collagen with an appropriate controlled release of bFGF was essential to achieve the *in situ* regeneration of adipose tissue even without preadipocytes.

Chapter 6

Controlled release of a chemokine, stromal cell-derived factor-1 (SDF-1) could be achieved with gelatin hydrogels of release carrier. Gelatin was chemically derivatized to give it different chemical properties of electric charge and hydrophobicity. Among the derivatives, succinylated gelatin (Succ) of an anionic charge was the most suitable for preparation of hydrogel in terms of SDF-1 release. The time profile of SDF-1 release from the hydrogel of succinylated gelatin could be controlled by changing the water content of hydrogel which can be modified with the conditions of hydrogel preparation. When evaluated after the subcutaneous implantation of Succ hydrogels containing SDF-1 or injection of SDF-1 solution, significantly stronger angiogenesis by the hydrogel was observed. The hydrogel implantation also enhanced the mRNA level of SDF-1 receptor at the site implanted. It is possible that the gelatin hydrogel enabled SDF-1 to locally release, resulting in an enhanced angiogenesis at the site implanted.

Chapter 7

The objective of this study is to evaluate the cellular contribution to the phenomenon of bone regeneration induced by the controlled release of bone morphogenetic protein-2 (BMP-2). Gelatin hydrogels (2 mg) containing BMP-2 (3 μg) with different water contents were subcutaneously implanted into the back of enhanced green fluorescent protein (GFP)-chimeric mice to induce the ectopic regeneration of bone tissue. The hydrogels containing BMP-2 could release BMP-2 in different time profiles. When evaluated radiologically and histologically, the ectopic regeneration of bone tissue was induced by the controlled release of BMP-2 from the hydrogels around

the hydrogel-implanted site. The number percentages of GFP- to osteocalcin-positive cells recruited into the bone tissue regenerated depended on the BMP-2 release profile. The higher the percentage, the stronger the bone regeneration. These findings indicate that bone marrow-derived osteoblast progenitor cells are recruited from the blood circulation by the BMP-2 release and consequently contribute to the ectopic regeneration of bone tissue. It is conceivable that the local concentration of BMP-2 modifies the recruitment profile of progenitor cells with an osteogenic potential around the site of BMP-2 released, resulting in regulated volume of bone tissue regenerated.

LIST OF PUBLICATIONS

Chapter 1. Y. Kimura, M. Ozeki, T. Inamoto, and Y. Tabata

"Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor."

Biomaterials, **24**, 2513-2521 (2003).

Chapter 2. Y. Kimura, T. Inamoto, and Y. Tabata

"Adipose tissue formation in collagen scaffold with different biodegradabilities."

J. Biomat. Sci. Polym. Ed., in press.

Chapter 3. In preparation.

Chapter 4. Y. Kimura, M. Ozeki, T. Inamoto, and Y. Tabata

"Time course of *de novo* adipogenesis in matrigel by gelatin microspheres incorporating basic fibroblast growth factor."

Tissue Eng., **8**, 603-613 (2002).

Chapter 5. Y. Hiraoka, H. Yamashiro, K. Yasuda, Y. Kimura, T. Inamoto, and Y. Tabata

"*In situ* regeneration of adipose tissue in rat fat pad by combining a collagen scaffold with gelatin microspheres containing basic fibroblast growth factor."

Tissue Eng., **12**, 1475-1487 (2006).

Chapter 6. Y. Kimura and Y. Tabata

"Controlled release of stromal cell derived factor (SDF) -1 from gelatin hydrogels enhances angiogenesis."

J. Biomat. Sci. Polym. Ed., in press.

Chapter 7. In preparation.

OTHER PUBLICATIONS

Kasper FK, Kushibiki T, Kimura Y, Mikos AG, Tabata Y. *In vivo* release of plasmid DNA from composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatin microspheres. J Control Release 2005;107:547-61.

Tambara K, Premaratne GU, Sakaguchi G, Kanemitsu N, Lin X, Nakajima H, Sakakibara Y, Kimura Y, Yamamoto M, Tabata Y, Ikeda T, Komeda M. Administration of control-released hepatocyte growth factor enhances the efficacy of skeletal myoblast transplantation in rat infarcted hearts by greatly increasing both quantity and quality of the graft. Circulation 2005;112:I129-I34.

Hamada Y, Katoh S, Hibino N, Kosaka H, Hamada D, Yasui N, Ozeki M, Kimura Y, Tabata Y. Effects of monofilament nylon coated with basic fibroblast growth factor on endogenous intrasynovial flexor tendon healing. J Hand Surg [Am] 2006;31A:530-40.

Kanemitsu N, Tambara K, Premaratne G, Kimura Y, Tomita S, Kawamura T, Hasegawa K, Tabata Y, M. K. Insulin-like Growth Factor-1 Enhances the Efficacy of Myoblast Transplantation With Its Multiple Functions in the Chronic Myocardial Infarction Rat Model. Journal of heart and lung 2006;25:1253-62.

Hirose K, Marui A, Arai Y, Nomura T, Kaneda K, Kimura Y, Ikeda T, Fujita M, Mitsuyama M, Tabata Y, Komeda M. A novel approach to reduce catheter-related infection using sustained-release basic fibroblast growth factor for tissue regeneration in mice. Heart and Vessels 2007;22:261–7.

Hori K, Sotozono C, Hamuro J, Yamasaki K, Kimura Y, Ozeki M, Tabata Y, Kinoshita S. Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing. J Control Release 2007;118:169-76.

Imamura M, Kanematsu A, Yamamoto S, Kimura Y, Kanatani I, Ito N, Tabata Y, Ogawa O. Basic fibroblast growth factor modulates proliferation and collagen expression in urinary bladder smooth muscle cells. Am J Physiol Renal Physiol 2007;293:F1007-17.

Kimura Y, Tabata Y. Experimental tissue regeneration by DDS technology of bio-signaling molecules. J Dermatol Sci 2007;47:189-99.

Kusanagi M, Matsui O, Sanada J, Ogi T, Takamatsu S, Zhong H, Kimura Y, Tabata Y. Hydrogel-Mediated Release of Basic Fibroblast Growth Factor From a Stent-Graft Accelerates Biological Fixation With the Aortic Wall in a Porcine Model. J Endovasc Ther 2007;14:785-93.

Lin X, Fujita M, Kanemitsu N, Kimura Y, Tambara K, Premaratne G, Nagasawa A, Ikeda T, Tabata Y, Komeda M. Sustained-Release Erythropoietin Ameliorates Cardiac Function in Infarcted Rat-Heart Without Inducing Polycythemia. Circ J 2007;71:132-7.

Hirose K, Marui A, Arai Y, Kushibiki T, Kimura Y, Sakaguchi H, Yuang H, Chandra BI, Ikeda T, Amano S, Tabata Y, Komeda M. Novel approach with intratracheal administration of microgelatin hydrogel microspheres incorporating basic fibroblast growth factor for rescue of rats with monocrotaline-induced pulmonary hypertension. J Thorac Cardiovasc Surg 2008;136:1250-6.

Mimura T, Amano S, Yokoo S, Uchida S, Yamagami S, Usui T, Kimura Y, Tabata Y. Tissue

engineering of corneal stroma with rabbit fibroblast precursors and gelatin hydrogels. Mol Vis 2008;14:1819-28.

Takemoto S, Morimoto N, Kimura Y, Taira T, Kitagawa T, Tomihata K, Tabata Y, Suzuki S. Preparation of Collagen/Gelatin Sponge Scaffold for Sustained Release of bFGF. Tissue Eng Part A 2008;14:1629-38.

Tsuji W, Inamoto T, Yamashiro H, Ueno T, Kato H, Kimura Y, Tabata Y, Toi M. Adipogenesis Induced by Human Adipose Tissue-Derived Stem Cells. Tissue Eng Part A 2009:15:83-93

Kimura Y, Tsuji W, Yamashiro H, Toi M, Inamoto T, Tabata Y. *In situ* adipogenesis in fat tissue augmented by collagen scaffold. J Tissue Eng Regen Med; submitted.

ACKNOWLEDGEMENTS

The present research was carried out under the continuous guidance of Dr. Yasuhiko Tabata, Professor of the Institute for Frontier Medical Sciences, Kyoto University. The author is deeply indebted to Professor Tabata for his constant guidance, encouragement, valuable discussion, and detailed criticism on the manuscript throughout the present work. The completion of the present research has been an exciting project and one which would not have been possible without his guidance.

The author wishes to express his special thanks to Dr. Masaya Yamamoto, Assistant Professor of the Institute for Frontier Medical Sciences, Kyoto University, for his thoughtful guidance, helpful suggestions, and intimate advice.

The author is also grateful to Dr. Takashi Inamoto of Kitano Hospital, The Tazuke Kofukai Medical Research Institute, Dr. Masakazu Toi, Professor of the Department of Surgery, Kyoto University, Dr. Hiroyasu Yamashiro, Assistant Professor of the Department of Surgery, Kyoto University, and Dr. Wakako Tsuji of the Department of surgery, Kyoto University, for their continuous discussion about the biology of adipose tissue and animal experiments, as well as providing preadipocytes.

The author wishes to express his sincere appreciation to Dr. Yasufumi Kaneda, Professor of the Department of Molecular Therapeutics, Osaka University, Dr. Katsuto Tamai, Associate Professor of Molecular Therapeutics, Osaka University, and Dr. Satoru Otsuru of Division of Oncology, The Children's Hospital of Philadelphia, for their significant advice and instruction of chimeric mice preparation.

The author also expresses his great thanks to Dr. Satoru Takemoto of the Department

of Plastic and Reconstructive Surgery, Kyoto University for the intimate discussion and helps on the animal experiment, Dr. Makoto Ozeki of Asahi Kasei Co. for the helpful advice and the instruction of hydrogel preparation, and Mr. Yosuke Hiraoka of Nitta gelatin Inc. for the significant support and intimate discussion.

The author is much indebted to the following students of Professor Tabata's Laboratory for their contribution to the present studies: Ms. Kaori Yasuda, Mr. Nobuhiko Miyazaki, Mr. Naoki Hayashi and Ms. Yuko Murakami.

The author likes to take an opportunity to extend his heartily thanks to Mrs. Miyuki Fukuma, Ms. Kyoto Bamba, Ms. Hisako Yoshida and other members of Professor Tabata's Laboratory for their kind help,

January, 2009

Kyoto

Yu Kimura